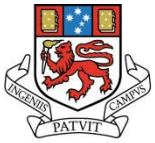

UNIVERSITY
OF TASMANIA

Bioinformatics, gene functional and germplasm physiology feature studies of *Zea may* ssp. *mexicana* L. under cold and other environmental stresses

by

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Submitted in fulfilment of the requirements for the Doctor of Philosophy

University of Tasmania

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Declarations

"The thesis contains no material which has been accepted for the a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright."

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This thesis was completed during the course of my enrolment in a Ph.D degree in the School of Land and Food at the University of Tasmania. This thesis contains no experimental results that have previously been presented for any degree at this or other institution.

This thesis contains one literature review chapter and four main research chapters. Results described in the two research chapters have been published (Chapter 2 and Chapter 3) and Chapter 4 is submitted for publication and Chapter 5 includes partial results for a paper to be submitted later.

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Abstract

The annual *Zea mays* ssp. *mexicana* L., a member of teosinte, has strong growth and regeneration ability, high tiller numbers, high protein and lysine content as well as resistance to many fungal diseases. It is a close wild relative of maize thus it can be effectively used in maize improvement. Most lines of *Zea mays* ssp. *mexicana* L. originated at high altitude in northern and central Mexico with a large spikelet and are adapted to acid soil. Since the best temperature for growing *Zea mays* ssp. *mexicana* L. ranges from 25 °C to 35 °C, it is considered to be more sensitive to cold/drought than to high temperature, especially during germination and early autotrophic growth stages. This project set out to detect the cold and/or drought tolerance of *Zea mays* ssp. *mexicana* L., using bioinformatics, gene functions and physiological features contributing to both stresses. The aims were to provide an effective approach to understand the responses of *Zea mays* ssp. *mexicana* L. to low temperature and drought. The understanding of molecular mechanisms for cold/drought tolerance can also help distinguish *Zea mays* ssp. *mexicana* L. from other tropical or temperate plants as well as provide useful references in improving abiotic stress tolerance of maize.

In a transcriptome study, we generated a seedling plant transcriptome at a sequencing size of 51.78 Gb of *Zea mays* ssp. *mexicana* L. under stress free, cold or drought stress conditions. A total of 414,232,462 high quality clean reads were used to conduct de novo assembly and annotation of genes without reference genome information. All of these reads were assembled into 251,145 transcripts (N50 = 1,269 bp) and 184,280 unigenes (N50 = 923 bp). A total of 3,504 up-regulated and 1,220 down-regulated genes were detected under cold stress and 532 up-regulated and 82 down-regulated genes were detected under drought stress. A total of 208 genes were affected by both cold and drought stresses. Examination of these genes suggested that the ABA dependent pathway, trehalose synthetic pathway and the CBF6 gene of the ICE1-CBF pathway may play important roles in both stress

tolerances of *Zea mays* ssp. *mexicana* L. Compared with the maize transcriptome database, GAs of *Zea mays* ssp. *mexicana* L. showed significant differences in regulating the responses to cold. These important functional genes identified in this study and the information on molecular mechanisms for cold and drought tolerance can provide useful references in improving abiotic stress tolerance of maize.

In gene functional studies, ZmmICE1 and ZmmSIZ2, two important cold related genes, were cloned and isolated from a cDNA library of RNA-Seq from cold-treated seedling tissues of *Zea mays* ssp. *mexicana* L. ZmmICE1, a novel MYC-type ICE-like transcription factor gene, enhanced freezing tolerance in transgenic *Arabidopsis thaliana*.

As a novel transcription factor, the ZmmICE1 protein localized in the nucleus and showed sumoylation when expressed in an *Escherichia coli* reconstitution system. ZmmICE1 showed ICE1-family characteristics, such as a highly conserved basic helix-loop-helix (bHLH) domain and a C-terminal region of ICE-like proteins. The other important gene ZmmSIZ2, a SUMO E3 ligase, was also isolated from the same treated plants. The biochemistry experiment showed sumoylation of the ZmmSIZ2 protein when expressed in an *Escherichia coli* reconstitution system. The structure analysis indicated that the deduced protein ZmmSIZ2 contained a highly conserved MIZ/SP-RING zinc finger domain for SUMO E3 ligase activity and bound to SUMO E2, a helix-extended loop-helix SAP (scaffold attachment factors SAF-A/B, Acinus, PIAS) domain for DNA binding and PHD domain.

In physiological studies, non-destructive chlorophyll fluorescence imaging technology was used to evaluate photosynthesis responses of three varieties of *Zea mays* ssp. *mexicana* L. under different cold treatments. Varieties showed significant differences in the damage caused by 5 °C and 15 °C treatments and the damage was dependent on treatment time. Additionally chlorophyll a and b showed the same tendency with imaging data.

This study provided useful information on bioinformatics, molecular biological and

physiological mechanisms of *Zea mays* ssp. *mexicana* L. tolerance of cold and drought stresses. The results could be used in maize genetic research and improving maize for cold and drought tolerance as well as reference data in NCBI database for other researchers.

Publications arising from this thesis

1. **Xiang LU,** Xuan Zhou, Yu Cao, Meixue Zhou, David McNeil, Shan Liang, Chengwei Yang. 2017 'RNA-seq analysis of cold and drought responsive transcriptomes of *Zea mays* ssp. *mexicana* L', *Frontiers in plant science*. vol. 8, no. 136. (Chapter 3).
2. **Xiang LU**, Lei Yang, Mengyuan Yu, Jianbin Lai, Chao Wang, David McNeil, Meixue Zhou, Chengwei Yang. 2017 'A novel *Zea mays* ssp. *mexicana* L. MYC-type *ICE*-like transcription factor gene *ZmmICE1*, enhances freezing tolerance in transgenic *Arabidopsis thaliana*', *Plant Physiology and Biochemistry*. Vol. 113, pp.78-88. (Chapter 4).

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List of abbreviation

F_m	Maximum chlorophyll a fluorescence
F_o	Minimum chlorophyll a fluorescence
F_v/F_m	Maximal quantum efficiency of PSII of dark adapted plants
F_v'/F_m'	Quantum efficiency of excitation energy trapping of PSII
NPQ	Non-photochemical quenching
PSII	Photosystem II
ΦPSII	Quantum efficiency of PSII open centers in the light adapted state
qP	Photochemical quenching
ETR	Electron transport rate
Chl	Chlorophyll content

Chapter 1 General introduction

1.1 The importance of pasture

The proportion of the livestock industry in intensive agricultural production is an important indicator, which can be used to measure the level of agricultural modernization of a country or a district. Forage is an extensive group, which includes all of the cultivated and grown wild plants, herbaceous plants, shrubs or arbors, that can be used to feed vegetarian livestock (Long, Yang & Qi 2007). It is not only indispensable to have extensive genetic materials for screening, improving and producing good cultivars, but also these are important materials for biodiversity study including classification, evolution and origin (Long, Yang & Qi 2007).Forage crops are generally divided into pasture or silage crops. Pasture and forage plants are therefore a most crucial component in agriculture and animal husbandry. They are critical for efficiently promoting the development of agriculture and forage production. In general, silage crops include forage maize, sorghum, oats, and others, in which stems and leaves are mainly taken as food by grazing and cradling (Barnes, Miller & Nelson 1995; Inoue, Fujimori & Cai 2007).

In most natural ruminant production system environments, 40%-90% of livestock are fed with forages. Livestock contribute 10%-45% to the gross domestic product (GDP) in the developing countries (Charmley 2001; Voisin *et al.* 2014). For feeding livestock, the use of pasture is a low cost feeding system as forage is the cheapest sources of nutrients (Peyraud *et al.* 2001). Animal products, such as meat, milk, egg and so on, are essential consumer goods for human life. The development of improved forages will therefore lead to improving people's living standards directly. Forage products account for 60% of intake in cattle and sheep feeding, 10%-15% in pig feeding and 3%-5% in chicken feeding (Givens *et al.* 2000; Minson 1990; Stobbs 1971).

Forage, including leguminous forage grass and gramineous forage grass, is one of the most important ecosystem components in the world having played a central role in human nutrition and culture development. According to their growth characteristics, forages can also be divided into annual forages, biennial forages and perennial forages (Nicotra *et al.* 2010). Perennial plants are more common than annual plants, and forage plants are essential for ruminant nutrition, especially perennial plants. Maize cropped for silage is the most extensively used annual plant. That is because corn is the third

most important crop in the world, which can be used for satisfying the needs of people and livestock (Barrière *et al.* 2003). The main direction of forage germplasm resources research is to focus on "extensive collection, proper preservation, deep study, aggressive innovation and full utilization" (Long, Yang & Qi 2007).

1.2 Current situation of forage in the world

World forage production can be divided into 5 main regions, North America, Europe, Asia-Pacific, Latin America, and the Rest of the World (ROW). Especially in some European countries, Australia, United States and New Zealand, pasture crops and pasture seeds industries are seen as traditional competitive industries. The development of a forage industry is indicative of a mature agricultural system, which is an essential component in national GDP (Gross Domestic Product). The revenue of the global forage seed market was valued at $85.06 billion (US) in 2013 (Newswire 2014). The prediction is that the global forage seeds market will increase 11.31% over the period 2013-2018 (Research & Markets 2014). North America is presently the largest forage seeds market and Brazil is forecast to be the fastest growing region in the Latin American seed market. However, the countries with the lowest increasing rate of pasture seed production include China and some developing countries. China is one of the countries which has the richest forage germplasm resources in the world. Since the 1960s, China has started to research forage germplasm (Inoue, Fujimori & Cai 2007). More than 15 provinces and districts have research plans for forage germplasm. These regions include the frigid zone, temperate zone, subtropical zone, tropical zone, Qinghai-Tibet Plateau and so on. According to published information (Hu, Hannaway & Youngberg 1992b), most of the elite germplasm is distributed in these areas, mainly herbs and a certain amount of semi-shrub forage plants. Therefore, the distribution of germplasm from these areas represents the whole country's geographical traits. Meanwhile, forage germplasm are mainly distributed in the Qinghai-Tibet Plateau, Yunnan-Guizhou Plateau, Hengduan Mountains, Hainan Island and arid desert. These areas contain 6,704 species, 1,545 genera, 246 families and 5 divisions (Hu, Hannaway & Youngberg 1992b; Long, Yang & Qi 2007).

However, there are few elite breeding cultivars suitable for planting in China, which leads to 80% of forage seeds still being imports (Inoue, Fujimori & Cai 2007). China is the second largest country of meadow in the world. The total area is about 400 million hectares, taking up 41.7% of the national territory area and being 4 times greater

than the national farmland area (Hu, Hannaway & Youngberg 1992a). Compared to traditional developed countries, the development and production level of pasture is still very low. The dominant problem is that the utilization of grassland resource is not well managed and efficient. Compared to other developed countries, which produce livestock using advanced methods, the livestock productivity per unit grassland in China low (Huan, Liu & Zhang 2005).

The consequence of the above observations is that development and utilization of forage and products in China may have great benefits and potential in the future. A major area of potential is the development of elite cold tolerant *Zea mexicana* (*Zea mays* ssp. *mays* L.) varieties which will improve forage wintering habit, enlarge forage planting area and increase forage yield in agriculture and livestock production in Southern China as *Zea mays* ssp. *mexicana* L. has a dominant status in this area. Meanwhile, development of high quality *Zea mays* ssp. *mexicana* L. varieties (high crude protein, high crude fat content, high crude fiber, high lysine content and disease tolerance) will have greatly increased economic value, which will also increase security of forage production, increasing the living standards and the level of consumer satisfaction.

1.3 Forage in China

China is one of the countries with the most diverse grassland resources in the world. As previously indicated it is the second largest country of meadow in the world (about 400 million hectares 41.7% of China), four times the area of farmland and 11.82% of the world's grassland third place after Australia and Russia (El-Nahrawy 2011). However, the dominant problem is that utilization of this grassland resource is not efficient. Compare to other developed countries, the livestock productivity per unit grassland in China is only 1/80 of New Zealand's, 1/20 of United states and 1/10 of Australia's (Huan, Liu & Zhang 2005).

Since the 1960s, China has started to research forage germplasm (Inoue, Fujimori & Cai 2007) with more than 15 provinces and districts (covering all the geographical areas of China) having research plans. According to the investigation results, most of the elite germplasm distributed in these areas are mainly herbs and a certain amount of semi-shrub forage plants. Forage germplasm are mainly distributed in the Qinghai-Tibet Plateau, Yunnan-Guizhou Plateau, Hengduan Mountains, Hainan Island and arid desert areas (Hu, Hannaway & Youngberg 1992a; Long, Yang & Qi 2007). China is

regarded to be the world center of origin region a number of crop and forage species. These have a number of wild populations and wild relative populations giving excellent cultivated forage species being widely distributed in China. There are wild species of many superior cultivated forages in China. These forages belong to 6,704 species, 1,545 genera, 246 families and 5 divisions (including 29 sub-species, 296 varieties and 13 forms) (Long, Yang & Qi 2007). A number of unique forage plants, such as 10 species in 7 families of Pteridophyta, 35 species in 6 families of Gymnospermae, and 273 species in 24 families of Angiospermae are distributed in China due to the diverse environmental status in its expansive territory (Table 1.1, Table 1.2). Among the 100's of species of cultivated forages in China, 30 species are sown on more than 10,000 ha (Table 1.3).

However, the scale of the forage industry is still small in China. The total yield of hay is 60,395,000 tons in 2008, which is no more than 1/10 of national requirements and only 2,800,000 tons of grass was traded commercially in 2008. The export volume was 180,500 tons, which accounted for 2.9% of the world trade; import volume was 19,800 tons which makes up 0.37% of world trade (China Animal Agriculture Association http://www.caaa.cn/; National Bureau of Statistics of the People's Republic of China http://www.stats.gov.cn/tjsj/; China Customs statistics http://www.chinacustomsstat.com). One of the most important factors is lack of available forage and forage seeds. There are few excellent forage seeds cultivated for production in China, 80% of the forage seeds are still imported into China. The main seed products are *Medicago sativa* and *Leymus chinensis*. Especially in the Guangdong area, the yield of forage seeds only rose 0.23% from 2001 to 2010. The production has decreased year by year, which is a major difference from the northern area. In 2012, the yield of milk of Guangdong province is no more than 0.4% of the national total volume, but the milk consumption accounts for 15% (Hannaway *et al.* 2005; Hu, Hannaway & Youngberg 1992a; Long, Yang & Qi 2007).

Table 1.1 Major families of forage plants of China

Family	Genera	Species	Subspecies	Variety	Variant type	Cultivar	% of total
Leguminoseae	125	1 157	6	69	4	3	18.48
Gramineae	210	1 028	15	98	3	4	17.12
Compositeae	136	532	1	5	-	-	8.03
Cyperaceae	24	350	1	7	-	-	5.34
Rosaceae	40	222	-	8	-	-	3.43
Chenopodiaceae	38	183	3	11	1	-	2.95
Liliaceae	20	150	-	5	-	-	2.31
Polygonaceae	11	135	-	8	-	-	2.13
Salicaceae	3	116	-	8	2	-	1.88
Others 237	938	2,479	3	84	3	-	38.32
Total 246	1,545	6,352	29	303	13	7	99.99

(El-Nahrawy 2011)

Table 1.2 Major genera of forage plants of China

Genus	Species	Subspecies	Variety	Variant types	Cultivar	% of total
Astragalus	276	2	14	1	1	4.39
Carex	181	-	3	-	-	2.74
Oxytropis	124	-	1	-	-	1.86
Poa	96	-	3	-	-	1.48
Roegneria	74	-	16	2	-	1.37
Salix	80	-	3	2	-	1.27
Allium	73	-	2	-	-	1.12
Polygonum	68	-	7	-	-	1.12
Caragana	65	-	7	-	-	1.07
Artemisia	66	-	2	-	-	1.01
Indigofera	63	-	2	-	-	0.97
Desmodium	54	-	8	-	-	0.92
Saussurea	53	-	-	-	-	0.79
Hedysarum	48	-	3	-	-	0.76
Others 1,531	5,031	27	232	8	6	79.13
Total 1,545	6,352	29	303	13	7	100.00

(El-Nahrawy 2011)

Table 1.3 Major forages and sown area

(Thousand ha, 1998)

Forage	Area	Form	Rank
Medicago sativa	1 804.7	Perennial	1
Astragalus sinicus	1 686.9	Perennial	2
Caragana koshinskii	1 108.7	Shrub	3
Astragalus huangheensis	653.2	Perennial	4
Vicia villosa	123.9	Biennial	12
Vicia sativa	98.9	Annual	14
Onobrychis viciaefolia	65.2	Perennial	17
Trifolium repens	31.7	Perennial	19
Oxytropis coerulea	28.7	Perennial	20
Trifolium pratense	28.2	Perennial	21
Stylosanthes guianensis	26.9	Perennial	22
Melilotus alba, *M. officinalis*	20.7	Biennial	24
Zea mays (forage)	570.5	Annual	5
Leymus chinensis	403.7	Perennial	6
Hordeum vulgare	358.7	Annual	7
Elymus sibiricus	230.3	Perennial	8
Lolium multiflorum	183.2	Annual	9
Avena sativa	155.7	Annual	10
Elymus dahuricus, *E. excelsus*	138.6	Perennial	11
Avena nuda	118.7	Annual	13
Setaria italica (forage)	80.0	Annual	15
Sorghum sudanense	77.2	Annual	16
Bromus inermis	22.5	Perennial	23
Secale cereale	20.1	Biennial	25
Lolium perrene	17.6	Perennial	26
Agropyron cristatum	14.4	Perennial	28
Dactylis glomerata	13.7	Perennial	29
Artemisia sphaerocephala	55.3	Sub-shrub	19
Raphanus sativus	17.0	Biennial	27
Amaranthus paniculatus	10.5	Annual	30

(El-Nahrawy 2011)

1.4 The utilization and development of forage products

Different types of forages have been widely used in productive activities in 3 main area; feeding livestock, protecting the environment and producing health food. Firstly, livestock husbandry is the indispensable source of meat, milk and other consumer goods for human life. In China, farm animal production plays an important role in rural areas and have grown rapidly since agriculture reform in 1978 (Li 2009). Meat output is an important indicator of the growth of China's livestock production, which indicates the population's standard of life. Forage, as the most important food source of livestock feed, has been extensively studied. In China, some forage products, such as forage bundles, forage pellets, forage powder, forage grain and leaf protein have already been used in livestock production (Long, Yang & Qi 2007). The most widely used product is forage bundles (bales) as it is the lowest in price and uses the simplest technologies. Forage powder is preferred for balancing the content of amino acids and complementing the feed content of vitamins and cellulose. With the development of the livestock industry, both of these sources have higher quality requirements for suitability to market. Leaf protein concentrate (LPC) are course protein products which provide a high-quality protein source. The general LPC product approach starts with harvesting fresh green plant material, grinding the plant tissue, squeezing out a protein rich juice and then heat the juice to precipitate and recover the protein (Kammes *et al.* 2011). It's addition to an animal diet can increase body weight of animals, improve the quality of meat, reduce forage consumption rate and improve its availability rate (Baloyi, Ayodele & Addo-Bediako 2013).

Secondly, for water loss and soil erosion, forage can be used to resolve these problems and improve the ecological environment and increase soil structure and soil nutrition. In red soil environments, forage, planted on hills, can reduce the surface runoff and soil erosion and retain soil effectively. Moreover, it will produce green manure, an organic fertilizer with excellent activity, which can promote the decomposition of organic materials, improve the soil quality, and increase the number of soil microorganisms and the activity of enzymes (Chen *et al.* 2015; Long, Yang & Qi 2007).

Thirdly, abundant amounts of proteins, fibers, vitamins and mineral elements are included in forage, which are good healthy products for human. In China, some processed forage products have been used widely, such as vegetable salad, hamburger and spring rolls, food additives added into wheat flour to make noodles and steamed

bread, medical products, cosmetics, drinks and so on (Long, Yang & Qi 2007).

Even though there are many achievements around forage use in China, there are still problems that need to be solved. These problems include: the genetic diversity of forage, forage genetic breeding, seeding creation and products processing etc. Therefore, developing biotechnologies and molecular breeding have a significant role to play in forage identification, screening, breeding, and a greater understanding of species traits and the utilization of forage production for commercial purposes and better meeting market requirements.

1.5 *Zea mays* ssp. *mexicana* L.

Zea mays ssp. *mexicana* L. *(syn. Zea mexicana)*, is a member of the teosinte. It is a wild grass native to Mexico and Central America, which is the closest wild relative of cultivated maize (*Zea mays* ssp. *mays* L.). It is an important genetic material for the study of maize genetics, quantitative genetics, molecular genetics, genome evolution and crop breeding (Figure 1.1and Figure 1.2) (Takahashi *et al.* 1999). Although teosinte has not yet been widely used in maize breeding, they have been regarded as having advantages as a genetic reservoir for the improvement of agronomic characteristics of maize and for improving teosinte itself for use (Cohen & Galinat 1984; Reeves 1950). Figures 1 and 2 below show the phenological/genetic extremes of teosinte.

Figure 1.1 Extremes in teosinte; ChalcoTeosinte. A Mexican-type teosinte with maize-like characteristics.

Figure 1.2 Extremes of teosinte; Guatemala Teosinte. The least maze-like race of teosinte, possessing a perennial-like growth habit, and resembling in some characteristics the genus *Tripsacum*.

The genus *Zea* includes two subgenuses (Figure 1.3): Section Luxuriants subgenus and genus *Zea* subgenus. Except for cultivated species of corn, all of the other species are uniformly named as teosinte. They are four species belonging to section Luxuriants subgenus and *Zea mays*, such as *Z. diploperennis* (2n=40), *Z. perennis* (2n=40), *Z. luxurians* (2n=20), *Z. nicaraguensis* (2n=20). *Zea mays* includes the subspecies maize (*Z. mays* L. ssp. *mays* 2n=20) and teosintes *Z. mays* L. ssp. *parviglumis* (2n=20), *Z. mays* L. ssp. *huehuetenangensis* (2n=20), and *Z. mays* L. ssp. *mexicana* (Schrader) (2n=20) (Almeida *et al.* 2011; Iltis & Doebley 1980); (Bonnett 1948; Doebley 2004; Nickerson 1954; Reeves 1950).

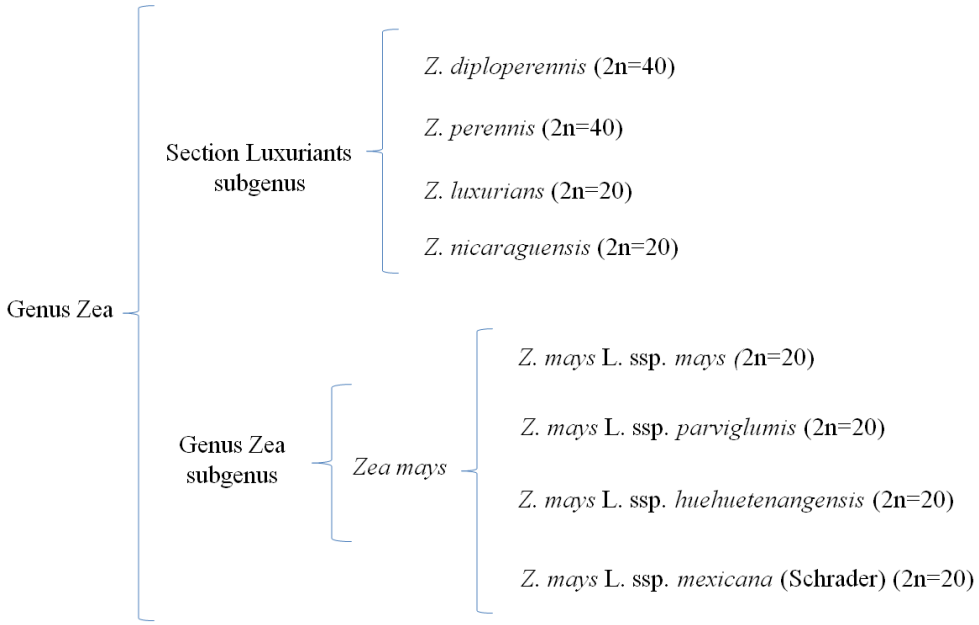


Figure 1.3 The relation of members of the Genus *Zea* found in Mexico.

According to what is known of the distribution of the genus *Zea*, teosinte is a wild grass native to Mexico and Central American and most of the teosinte species and subspecies have narrow geographic distributions consisting of only a few local populations except for ssp. *mexicana* and ssp. *parviglumis*, which have a wide distribution in Mexico. *Z. diploperennis* is a diploid perennial teosinte from Jalisco, Mexico; *Z. perennis* a tetraploid perennial teosinte from Jalisco, Mexico; *Z. luxurians* an annual teosinte from Central America; ssp. *parviglumis* can live at mid-altitude (about 400~1800 m) in southwestern Mexico and has a small spikeleted trait. Ssp. *huehuetenangensis* is an annual teosinte just be found in the province of Huehuetenango in western Guatemala; ssp. *Mexicana* is found at high altitude in northern and central Mexico (about 1600~2700 m) with a large spikeleted trait (Figure 1.4) (Fukunaga *et al.* 2005).

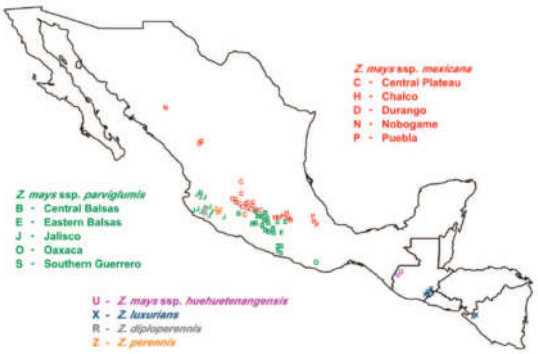


Figure 1.4 The geographical distribution of the teosinte populations. Since many accessions come from geographically very close locations they overlap on the map (Fukunaga *et al.* 2005).

Studies showed that *Zea mays* ssp. *mexicana* L. and cultivated maize have normal chromosome pairing in meiosis and general fertility. The frequency of the chromosome crossing between maize and teosinte is similar to that of maize intercrossing (Doebley 2004; Wang *et al.* 2008). Compared with maize, there are two major differences between *Zea mays* ssp. *mexicana* L. and cultivated maize. The first is the kernels of *Zea mays* ssp. *mexicana* L. are packaged by the fruitcase which is formed closely around them. However, the maize kernels are naked. The second is *Zea mays* ssp. *mexicana* L. has more tillers and long lateral branches (Wang *et al.* 2008). The annual *Zea mays* ssp. *mexicana* L. has some valuable traits, such as strong growth ability and regeneration ability, more tillers, high protein content in the kernel and dominant resistance to many fungal diseases (Fang *et al.* 2012; Hufford *et al.* 2012; Mangelsdorf 1961).

Zea mays ssp. *mexicana* L. was first introduced into China from Japan in 1979. Planting areas have covered Jiangxi, Zhejiang, Fujian, Guangxi, Sichuan, Hunan, Jiangsu province, etc. The yield of *Zea mays* ssp. *mexicana* L. is far beyond other kinds of forage grass in these areas. The growth period is 180~330 days, the plants can adapt to acid soil and the best growing temperature's range from 25 °C to 35 °C. But it is difficult for the plants to face low temperature stress, stopping growing below10 °C and death if temperatures fall under 1 °C. The number of tillers are about 30~60, and the most is more than 90 branches. As a silage plant, *Zea mays* ssp. *mexicana* L. can be harvested 7~8 times in one growing season. In general, the total harvest yield (leaves and stem) can reach up to 150,000~450,000 kg/hm^2. Harvest products can be used to feed livestock directly or be reserved as silage and hay. The quality of stems and leaves are excellent and have a sweet taste. The content of crude protein, crude fat, crude fiber and lysine are abundant, 3.8%, 2%, 22.73% and 0.42% respectively. Especially the content of lysine is almost equal to maize, which enhances the digestion and utilization. As forage maize, it has a wide scope of application, which can be fed not only to livestock but also to include rabbit, fish and geese etc. (Fukunaga *et al.* 2005; Niazi *et al.* 2014; Wllkes 1977).

Chapter 2 Literature review

2.1 Physiological and ecological studies of cold resistance in *Zea mays* ssp. *mexicana* L.

About 1/3 of the total land area on the earth is free of ice and 42% of land undergo temperature extremes of −20 °C or less. In such areas, plants have to develop a serious of mechanisms to survive variations in environmental status (Ramankutty *et al.* 2008). Low temperature (LT) stress is one of the most extensive abiotic stress factors affecting the yield, quality and distribution of plants. The range of low temperature injury of plants includes chilling injury (0-15 °C) and freezing injury (<0 °C), which are common temperatures in nature and damage factors for plants. Some plant species can increase the ability for cold acclimation by responding to low temperatures with an active series of physiological and molecular responses (Theocharis, Clément & Barka 2012). Therefore, the need for plants to have cold tolerance initially is not necessary, cold tolerance develops from some plants being exposure to low temperature conditions.

Generally, temperate regions plants, such as *Arabidopsis*, have a certain degree of chilling tolerance and can increase the extent of freezing tolerance in response to chilling and non-freezing temperatures. Conversely, plants of tropical and subtropical districts are usually sensitive to low temperature stress. The former plants have cold acclimation; but others often lack this mechanism (Hincha & Zuther 2014). When plants perceive a low temperature environment, a series of physiological and ecological responses will be changed, which play an important role in plant characteristics and depend on the plant's genetic background potential (Martiniello & Teixeira da Silva 2011).

Low temperature exposure can lead to formation of ice crystals in the cell and cause alterations, which includes the change of cell membrane structure and lipid composition. These changes can result in losing function, cellular leakage of electrolytes and amino acids, a transferring of electron flow pathways, alterations in protoplasmic streaming and redistribution of intracellular calcium ions (Seo *et al.* 2010). In addition, low temperature can also change protein content and the activity and the reaction speed of enzymes and metabolism (Ruelland & Zachowski 2010). Ultrastructural changes of cell components, such as plastids, thylakoid membranes and the phosphorylation of thylakoid proteins, and mitochondria of plant will be changed also (Leyva *et al.* 1995; Matonyei *et al.* 2014; Matteucci *et al.* 2011). The reaction

center and light-harvesting antennas of the basic photosynthesis membrane system, lie on the chloroplast thylakoid phospholipid bilayer membranes. These carry out the process of transforming light energy into biochemical usable chemical potential energy (ATP) and redox potential energy (NADPH) (Ensminger, Busch & Huner 2006). Under low temperature stress, the structural stability of the lipid bilayers is reduced. Especially the photosynthesis PSII reaction place can be harmed under this stress (Huner *et al.* 1993).

According to previous research results, more than 70% of 400 types of metabolite product will be induced by low temperature (Cook *et al.* 2004a). Soluble proteins, soluble sugars and proline are the most important metabolites for osmoregulation. These osmoregulation and vitamin E (antioxidant) changes also can reduce plant injury under low temperature (Maeda *et al.* 2006). Cells can reduce the damage of chilling injury or freezing injury by improving sugar content, increasing antioxidants, taking mechanical action and inducing molecular chaperone chemicals (Leyva *et al.* 1995). Meanwhile, metabolic processes of cell membranes can also be changed by low temperature, such as photosynthesis and respiration (Ryan 1991).

2.1.1 Chlorophyll fluorescence

Photosynthesis is the most important and complex chemical reaction on the earth, which offers essential energy for life of photosynthetic organisms. Green plants are oxygen producing photosynthetic organisms. They can release oxygen (O_2) by splitting H_2O with absorbed solar energy and converting carbon dioxide (CO_2) to sugars. The O_2 will be used to allow the breath of life to animals and the sugar will be used to fuel their life activities. Therefore, photosynthesis is the basis of the cycle of mass and energy fluxes in the earth's ecosystem (Hopkins & Hüner 1995). The progress of photosynthesis includes a serious of processes, such as absorbing solar energy, energy conversion, electron transfer, ATP synthesis and CO_2 fixation. More than 66 steps exist in these complex processes. All of these processes can be divided into two main reactions: the dark reaction and the light reaction. Absorbing solar energy and ATP synthesis compose the light reaction, the reaction site is at the thylakoid membrane and 4 types of protein complexes are on the thylakoid membrane which takes part in this reaction, such as PhotosystemII (PSII), Cyt b_6/f, PhotosystemI (PSI) and ATP synthase. The dark reaction process is the CO_2 fixation process, which occurs in the chloroplast stroma or prokaryotic cell plasma (Chaves, Flexas & Pinheiro 2009; Rabinowitch 1956).

For crop tolerance breeding and large-scale industry crop selection and identification, developing high quality and cost effective measuring of the phonological response is the most important research tool. With the development of measurement technologies, chlorophyll fluorescence (ChlF) measurement parameters have become an effective diagnostic mode for plant responses, which has greatly promoted the development of plant research in physiology, ecophysiology, phytopathology, etc. (Murchie & Lawson 2013). These measurements are not only for understanding the photosynthetic mechanisms but also especially useful in studying plant photosynthesis under stress. The measurements are a rapid method to assess photosynthesis response (Kooten & Snel 1990). This technology has a short history. It was first applied to experiments by Kauttsky and Hirsch (Kautsky & Hirsch 1931). They observed and recorded the phenomenon of ChlF by naked eye observations initially. After that, this technology has progressed extensively. By the 1980s, this technology had been transformed greatly in experiments (Bradbury & Baker 1981; Ögren & Baker 1985; Schreiber, Schliwa & Bilger 1986). The benefits of these changes have made ChlF much easier to measure under changing irradiance and allow us to explain the basis of ChlF by using the concept of quenching due to photosynthetic electron transporter non-photochemical quenching.

ChlF means chlorophyll molecules change to the first excited level from the ground state by absorbing a light quantum, which is an energy unstable status. There are three kinds of energy transformation results for the first excited level: ① Heat energy loss from the first excited level transfer to the second excited level; ② Heat energy loss from the first excited level to ground status; ③ Emission fluorescence from the first excited level return to the ground status. Among all of them there is a competitive relationship. Under normal conditions, most of the emission fluorescence comes from chlorophyll a in PSII (Krause & Weis 1991). Therefore, the ChlF index can be used to detect photosynthesis and heat energy transformation. During the ChlF analysis procedure, the basic parameters are Minimal fluorescence (dark) (F_0); Maximal fluorescence (dark) (F_m); Minimal fluorescence (F_0'); Maximal fluorescence (F_m'); Variable fluorescence (F_v); Maximal photochemical efficiency (F_v / F_m); Photochemical quenching coefficient (q_P); Non-photochemical quenching coefficient (q_N) and Fluorescence yield, which is mainly dependent on the degree of opening of PSII response center (RC) (Table 2.1). The reaction center situation can be estimated

by the changes of F_0. Under stress status, F_0 will reduce as PSII loses energy via heat loss. Conversely, F_0 increase indicates that PSII RC system has been inactivated or damaged (Demmig & Björkman 1987). Especially, under low temperature stress, the efficiency of PSII will decrease, which leads to the value of F_v / F_m reducing. That reduction is because low temperature harms the photochemical reaction of PSII. ChlF emission can detect this harm non-invasively by measuring the response of thylakoids membranes with sustained low-temperature treatments (Baker 2008; Gremer *et al.* 2012). ChlF index is already used as an effective tool for detecting low temperature response in many plants and crops, such as barley, wheat, rice, *Arabidopsis,* banana, etc (Kalaji *et al.* 2011; Mathur *et al.* 2011; Mishra *et al.* 2011).

Meanwhile, the change of F_v / F_m also indicates a type of plant protective response. The change of ChlF parameters also relates to the season in field screening. F_v / F_m and low temperature damage have a linear relationship. If the degree of damage shows an increase, the value of F_v / F_m will ascend. Therefore a relationship between the low temperature semi-lethal temperature (LT_{50}) and F_v / F_m can be produced (Rose & Haase 2002). The F_v / F_m value is also negatively related to electrolyte leakage, which is also an important index of plant cold resistance ability. Moreover, the reduced carbon exchange rate (CER) with reduced F_v / F_m value also means these two have a linear relationship (Gilles & Binder 1997). The ChlF technology was first applied to selecting plant cold traits by Australia scientists (Smillie & Hetherington 1983). They used dark conditions to treat plant leaves for 1h, and then treated them for different times with 0 °C, in the dark or with green light. The results showed that the maximal rate of the induced rise in ChlF and the actual time of F_R (Maximum rate of ChlF rise) was decreased by 50% in leaves at 0°C. It was demonstrated ChlF is an available index of chilling tolerance (Smillie & Hetherington 1983). This index is already in use in plant breeding as it is a rapid and easy method to use. Therefore, the ChlF technology can be used to identify the ability of plants to have cold resistance.

Table 2.1 Definitions of basic parameters of ChlF.

Fluorescence intensity indicators		
F_0	Minimal fluorescence (dark)	Fluorescence intensity with all PS II reaction centers open while the photosynthetic membrane is in the non-energized state
Fm	Maximal fluorescence (dark)	Fluorescence intensity with all PSII reaction centers closed, all non- photochemical quenching processes are at a minimum. This is the classical maximum fluorescence level in the dark or low light adapted state.
F_0'	Minimal fluorescence	Fluorescence intensity with all PS II reaction centers open in any light adapted state
Fm'	Maximal fluorescence	Fluorescence intensity with all PS II reaction centers closed in any light adapted state
F_v	Variable fluorescence (dark)	Maximum variable fluorescence in the state when all non-photochemical processes are at a minimum
Fluorescence quenching parameters		
q_p	Photochemical quenching	$(F_m'–F')/(Fm'–F_0')$
q_N	Non-photochemical quenching	$1–(F_m'–F_0')/(F_m– F_0)$

(Baker 2008; Kooten & Snel 1990)

2.1.2 ChlF parameter of environmental stress

Assessments of photosynthesis is a good indicator for predicting plant stress. Because photosynthesis is sensitive to changes of environmental conditions, especially low temperature (Perks, Osborne & Mitchell 2004a). ChlF as a non-destructive, rapid and effective method has been used as a most reliable indicator of the performance in plants and crops products. Until now, it has been widely used to detect differential stress responses. Such as water stress, heat stress, salt stress, chilling stress etc. (Sayed 2003b).

Low temperature injury can be divided into freezing and chilling:

At freezing temperature, leaf metabolism is greatly inhibited and recovers slowly, which is caused by the photo damage of PSII (Baker & Rosenqvist 2004). ChlF

parameter F_v / F_m ratios can be used to measure the status of the Photosystem II (PSII) reaction center for detecting freezing tolerance (Sayed 2003b). In freezing studies, F_v / F_m was linearly related to visual needle damage and short-term survival after freezing stress. The parameter is available to accurately predict cold hardiness (Perks, Osborne & Mitchell 2004b). Moreover, it can be used to estimate the critical temperature for 50% needle damage after freezing (Lindgren & Hällgren 1993). Analysis of freezing injury of Palm showed that there is a strong positive correlation (r^2 = 0.94) between injury index F_v / F_m values and extent of injury (Equiza & Francko 2010). In a potted Oleander study, they found that after freezing treatment, non-photochemical quenching (NPQ) and the maximum photochemical efficiency of photosystem II (F_v / F_m) were decreased (Julián *et al.* 2011).

Chilling injury influence the growth of the plant and development of cereals. The range of inducing temperatures is from 0 to 15 °C. According to the extent of the injury, it may be classified as primary or secondary chilling injury. The former indicates metabolic dysfunctions which can reverse when the temperature returns to the normal range, the latter happens irreversibly when metabolic dysfunctions occur (Sayed 2003b). Crops in temperate zones are generally much more tolerant of falling temperatures than subtropical and tropical species. Moreover, chilling inhibited metabolism can more rapidly recovery when temperatures rise in these plants. However, many temperate regions crops are also very sensitive to low temperature if the plants have not experienced effective low temperature acclimation. The growth and physiology of crops of subtropical and tropical districts can be influenced by exposure to low temperatures as high as 15 °C (Baker & Rosenqvist 2004). In general, compared with mature plants, the seedling growth stage is more sensitive to chilling stress and chilling injury leads to plant loss of viability. Moreover, flower organs will be more seriously influenced by chilling stress at the reproductive growth stage (Sayed 2003b). Chilling injury will lead to down-regulation and photo damage to PSII. This inhibition includes reduced stomatal aperture, membrane lipid changes and reduced CO_2 fixation and electron transport of PSII (Agati *et al.* 1996). Chilling stress leads to a decrease of F_v / F_m, which has been widely used to screen differential chilling tolerance of crops.

In many districts of the world, drought from climate change is a major abiotic stress factor that severely affects food production. It leads to high temperatures and reduced water supply for crops (Mishra & Singh 2010). Improving drought tolerance with agronomic and physiological phenotype trait selection is important to reduce the

impact of water deficit on crop yield in a breeding program (Tuberosa & Salvi 2006). In many species, ChlF has been used as a probe for detecting and predicting the degree of drought response (Ögren 1990; Oraguzie *et al.* 2007; Razavi *et al.* 2008). In a barley study, they found that the values of chlorophyll content, F_0, F_v / F_0 and F_v / F_m in drought tolerant genotypes were significantly higher than drought sensitive genotypes under drought stress (Li *et al.* 2006). ChlF parameters as indicators of membrane injury can be evaluated. The F_v / F_m ratio is employed to assess changes in the primary photochemical reactions of the photosynthetic apparatus after dehydration (Kocheva *et al.* 2004). In addition, ChlF values can be used as phenotype information combined with population genotype information for detecting crop ChlF related QTLs at specific drought stages (Guo *et al.* 2008).

Salinization of soils is an extensive problem in crop production systems. The initial effects of increasing soil salinity are similar to drought effects when plants are exposed to drought (Mckersie & Ya'acov 1994). F_v / F_m values were found to be unaffected by salinity treatments, whereas the overall or actual efficiency of photochemical energy conversion (actual quantum yield $\Delta F / F_m$) reduced with increasing salinity. Non-photochemical quenching increased significantly with increasing salinity level in salt-sensitive cultivars (Dionisio-Sese & Tobita 2000). F_v / F_m was also demonstrated to have a lack of change when maize was grown in high concentrations of NaCl (Shabala *et al.* 1998). Salt stress-induced effects in cereal crops include reduction of water uptake by roots due to perturbation of osmotic equilibrium, and inhibited cell expansion, reduced respiration and reduced cytokinesis (Blum & Johnson 1992; Sayed 2003a).

Heat stress, may also limit the growth of crops and their yield. The most dominant heat stress traits are inactivation of PSII and thylakoid disorganization. The value of F_0 has a sharp rise indicating the critical temperature which leads to PSII inactivation. Heat stress also enhances respiration and causes plants to consume saccharide reserves faster than photosynthesis replaces them (Baker & Rosenqvist 2004). In tomato studies, it was found F_v / F_m and F_v'/F_m' decreased in the heat-sensitive genotypes. F_v'/F_m' was better that F_v / F_m for predicting crop heat-shock sensitivity (Hameed *et al.* 2015). CO_2 assimilation and electron transport are both inhibited with heat stress, measurements of F_q'/F_m' and F_v'/F_m' also have potential for use in screening to identify tolerance to high temperatures (Baker & Rosenqvist 2004).

2.1.3 The application of ChlF in cereal crops research

Arabidopsis thaliana as a model plant, is always a species of choice for testing the newest technology application. Cold tolerance as a result of cold acclimation was measured by using a non-invasive method (ChlF). Nine *Arabidopsis thaliana* accessions (Cvi, Can, C24, Co, Col-0, Nd, Ler, Rsch and Te) were measured for cold sensitivity and tolerance. Six week intact whole plant seedlings were treated by 3 types of treatments: non-acclimated; cold acclimated 4°C for two weeks; sub-zero temperature treated -4°C for 8 h in the dark. A combined image and statistics analysis 3 replications showed that the ChlF parameter: maximum quantum yield of PSII photosystems (F_v / F_m) and fluorescence decrease ratio (R_{FD}) had some significant differences. For the cold sensitive accessions (Co, C24, Can and Cvi), ChlF transients were basically consistent across the 3 conditions. For intermediate cold tolerant accessions (Ler and Nd) and cold tolerant accessions (Col, Rsch and Te), ChlF emission had significant changes. Effective quantum efficiency of PSII photochemistry (ΦPSII) was significantly higher in cold acclimated *vs.* non-acclimated except for Cvi. Combining images analysis, the different cold sensitivity of the nine species was well discriminated and characterized. This experiment indicated that ChlF is a valuable method for high-throughput detection of cold tolerance of whole plants (Mishra, Heyer & Mishra 2014).

In cereal crop studies, different types of important crops have been used for high throughput screening with chlorophyll fluorescence imaging ChlF parameters and visual imaging for large scale detection of traits and marker assisted selection breeding. This is a powerful technique as advanced phenotyping parameters have been widely used on crop species for speeding up breeding progress. Especially for some complex traits, such as photosynthesis, variants are more easily detected for cloning by using high-throughput screening (HTPS) of phenotypes and genotypes. Moreover, HTPS can replace traditional labor and cost intensive work. Except for initially preparing the available population for testing, harvesting accurate phenotypic information is always the decisive factor in successful breeding. Among the many available technologies, chlorophyll fluorescence imaging (CFI) technology is an optical, rapid, non-contact, non-destructive and repeatable method that can be used in any growth stage of the plant allowing the physiological state of plants to be measured with different treatment responses (Harbinson *et al.* 2012). Combining this with genotype data, linkage mapping and association mapping can be used to detect QTLs locations on crop chromosomes.

Photosynthesis has an essential function on plant yield research and it has an important significance in crop genetics and breeding for improved productivity with reduced inputs in the future (Flood, Harbinson & Aarts 2011). CFI offers an option to analyse a number of steps of the photosynthetic process. With present technology about 10^3 plants can be measured several times per day just by one camera. The huge amount of image management required to deal with this data requires advanced image software and better physiological models for analyzing (Harbinson *et al.* 2012).

In a winter and spring oats frost damage study, ChlF was used to evaluate the cold acclimation and freezing damage. 12 winter and 3 spring oats (*Avena sativa* L.,) were used as experimental materials and were cold acclimated and treated with freezing treatments. A dominant reversible decrease in F_v / F_m was found in all genotypes during acclimation to low, non-freezing temperatures, which is a rapid and effective method to detect crop low temperature tolerance. It has been identified ChlF is a more sensitive and precise method than others and is highly correlated with field-evaluated frost damage (Rizza *et al.* 2001). Crown rust (*Puccinia coronata*) infected oat leaves give rise to heterogeneous changes in photosynthesis as identified by quantitative imaging of chlorophyll fluorescence (Scholes & Rolfe 1996). Moreover, salinity tolerance physiological responses also can be detected by ChlF method (Zhao, Ma & Ren 2007). Cold acclimation and freezing tolerance of winter and spring oats (*Avena sativa* L.) were rapid evaluated and measured by F_v / F_m (Herzog & Olszewski 1998).

In wheat studies, nitrogen deficiency and water deficit strongly reduced the photosynthetic activity. ChlF parameter F_v / F_m showed that the affect is more significant from the low-N than the high-N treatment. Nitrogen deficiency leads to reduction of the total ChlF content and increases the Chl a/b ratio (Shangguan, Shao & Dyckmans 2000). Grain yield is one of the important selection criterion for durum wheat (*Triticum durum* Desf.). The ChlF measurement method showed that the growing environment has a strong influence on yield and all the fluorescence parameters (Araus *et al.* 1998). In a doubled haploid population of 94 lines from the wheat cross Chinese Spring × SQ1, 116 Quantitative trait loci with ChlF parameters were located on all chromosomes except 7B; 39 and 3 QTLs were identified for pigments and plant productivity traits which were mapped separately. 14 chlorophyll content and grain weight per ear QTLs were detected on chromosome 6B. All of that will provide key traits genetics information for future breeding (Czyczyło-Mysza *et al.* 2013). Moreover, 37 heat tolerance QTLs were also located by ChlF kinetics parameters and 5 QTLs

regions significantly associated with response to heat stress of wheat were identified (Azam, Chang & Jing 2015; Talukder *et al.* 2014).

In maize studies, ChlF parameters have been be used to evaluate differential heavy metal toxicity of maize plants at differential stages (Adam & Murthy 2014; Da Silva *et al.* 2012; Gouveia-Neto *et al.* 2012; Marques & do Nascimento 2013). Cold stress is a key environmental factor, the changes in ChlF signals have help detect, improve and understand cold induced response mechanisms in maize studies (Rodríguez *et al.* 2014; Rodríguez *et al.* 2013). Effects of nitrogen fertilization of maize were also be measured by ChlF parameters for improving N utilization and gas exchange (Akram *et al.* 2011; Wu *et al.* 2013). QTLs genetic analysis was also applied in maize genetics breeding in field environments using morphological traits (Cai *et al.* 2012; Šimić *et al.* 2014). Under water-limited or drought condition, photosynthetic performance was detected by ChlF for comparing differential maize inbred lines (Lepeduš *et al.* 2012; Liu *et al.* 2012). In addition, cold related QTLs genetics loci were found (Rodríguez *et al.* 2014). Changes in fluorescence kinetics were used also for testing the effects of high temperature in maize (Xu *et al.* 2011).

In rice research, photochemical properties of flag leaves of different rice species were detected by ChlF transients (Zhang *et al.* 2015). F_v / F_m was also used to evaluate somaclonal variations related to improved chilling tolerance in rice (Bertin, Bouharmont & Kinet 1997). Non-photochemical quenching (NPQ) capacity, with regulates energy conversion in photosystem II and protects plants from photoinhibition was induced by medium and high light intensities or osmotic stress in rice leaves of rice cultivars (Kasajima *et al.* 2011; Li *et al.* 2015). The dynamic change of microelement/microelement and ChlF were used to determine photosynthetic system changes and the changes in grain for improving rice quality (Shrestha, Brueck & Asch 2012; Zhang *et al.* 2014). In a field environment, ChlF provides reference data for field growth and yield management, such as flooding/waterlogging stress, field day and night temperature, heat and drought stress and toxicity etc. (Gu *et al.* 2014; He *et al.* 2013; Kumar, Vijayalakshmi & Vijayalakshmi 2014; Šebela *et al.* 2015).

2.2 Transcriptome and Gene Expression Profile

2.2.1 The second generation sequencing technology

In the past 30 years, the Sanger DNA sequencing method as a gold standard has been regarded as the dominant approach. It depends on the dideoxy method. DNA

polymerase elongates the complementary strands with short random primers. The additive four differently labeled dNTPs can be separately detected in chain termination and therefore their presence enables the identification of the unknown DNA base sequence at that point (Men *et al.* 2008). In contrast to the Sanger sequencing method, pyrosequencing uses synthesis sequencing technology. Incorporation of nucleotides during DNA sequencing is monitored by luminescence, as after nucleotide binding a light signal is generated. Only one of four incorporated nucleotides can cause a signal, which leads to single nucleotide polymorphisms being detected by this method (Ahmadian *et al.* 2000).

In biological research, DNA sequencing is a necessary step and the sequence quality is a direct determinant of the study result quality. Formerly mRNA expression was identified by microarray or real-time PCR techniques. However, the issues with these two methods are comparative low precision and quite expensive costs. Thus it is difficult to use to detect gene expression on a genome-wide scale (Heller 2002; Mutz *et al.* 2013). Early dideoxy chain termination technology has been replaced by pyrosequencing platform use to increasing technological requirements. The human genome project spent more than 15 years and millions of dollars. However, the new technique means it would now take 8 days and the cost cut to $100,000 for finishing same project (Venter *et al.* 2001). The first massively parallel pyrosequencing platform based on the sequencing-by-synthesis (SBS) technology was used in 2005. It created a new era of high-throughput genomic analysis (Sanger, Nicklen & Coulson 1977) . Especially in the past ten years, NGS (Next generation sequencing) of several different organisms has altered genomics research and created highly successful applications. Researchers may conduct experiments that were previously not affordable (Voelkerding, Dames & Durtschi 2009). With the development of genomics, biological informatics studies and NGS technologies, sequencing information has become more and more an important research hotspot. Because large-scale sequencing results can provide massive amounts of nucleotide traits information, such as SNP (Single nucleotide polymorphism), small insertions or deletions (indels), copy number variations (CNVs), SV (Structure of variation), and microarray chips results (Daniel, Paniz-Mondolfi & Monzon 2015; Jia & Zhao 2012). All of that can be used to help biologist better understand the functions of genes at the genome level.

Many technologies have been developed to detect and quantify transcriptome information, including hybridization or sequence-based methods. Since the first

nucleotide sequence of a gene was developed by Holley Maxam, Gilbert and Sanger who among others have promoted and developed this technology (Holley 1975; Maxam & Gilbert 1977). In 2004, sequencing technology faced a great revolution as massively parallel sequencing platforms emerged (Mardis 2008). This has greatly increased now with the development of second generation sequencing technologies platforms. Currently, the NGS market is dominated by three maim platforms: the FLX pyrosequencing system from 454 Life Sciences (Roche company), the Illumina Genome Analyser (developed initially by Solexa), and the AB SOLiD (Sequencing by Oligo Ligation and Detection) system (Life Technologies), which were developed at the end of 1990s and commercialized around 2005 (Ansorge 2009). All three platforms depend on parallel sequencing of DNA fragments, which bring a number of short sequences, namely "reads" or "tags". There are hundreds of thousands of reads produced by the FLX system. However, the total number of reads from the SOLiD and Illumina systems are far more than from the FLX system and the reads length varies from 30-100 bp. Updating of the technology lead to numbers and lengths of sequence reads being tremendously increased (Mardis 2008). The flowcharts of the three differential types of sequencing show basic similarity for producing and analyzing of sequencing libraries (Figure 2.1). Firstly, the RNA samples have to be adapted to the quality requirements of the platform. The RNA is always digested to small fragments (typically <500 bp). Secondly, DNA adapted with special sequences are linked to both ends and then sequenced in parallel. Thirdly, libraries are constructed for the different samples. Fourthly, comparing and cleaning data and evaluating the quality of data is carried out. Finally, differential expressed genes analysis, unigenes functional annotation, gene metabolism pathways and gene express profile analysis are carried out.

In recent years, a new strategy of single molecule sequencing is booming. Helicos Biosciences has introduced its version of single-molecule sequencing (tSMS) (http://www.helicosbio.com/), the Helicos Genetic Analysis System (http://www.pacificbiosciences.com). Pacific Bioscience also introduced its Zero-Mode Waveguide based SMRT technology (Zhou *et al.* 2010) (Table 2.2).

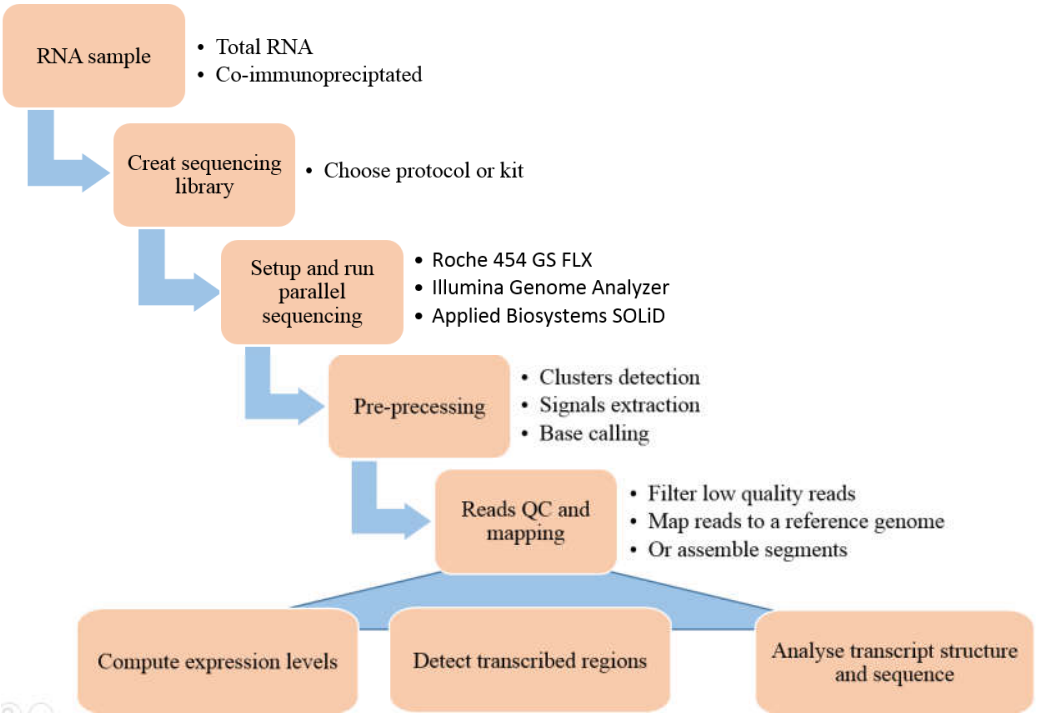


Figure 2.1 Flowchart of a typical RNA-Seq experiment

Table 2.2 Comparison of NGS platforms.

	Roche 454 GS FLX	**Illumina Genome Analyzer**	**Applied Biosystems SOLiD**	**Sanger**	**Helicos Biosciences Heliscope**	**Pacific Bioscience RS system**
Sequencing method	Pyrosequencing	Reversible dye terminators	Sequencing by ligation	Dye terminators	Sequencing by synthesis	Sequencing by synthesis/real time
Read lengths	400 bp	36 bp	35 bp	800 bp	32 bp	1100 bp
Sequencing run time	10 h	2.5 days	6 days	3 h	8 d	N/A
Total bases per run	500 Mb	1.5 Gb	4 Gb	800	37 b	13 b

(Voelkerding, Dames & Durtschi 2009; Zhou *et al.* 2010)

In a range of different fields of study, the next generation sequencing technologies have made great progress (Table 2.3). These fields include: genome sequence (whole-genome re-sequencing for indels, copy number and structural variations, *de novo* genome sequencing, targeted sequencing for discovery of mutations or polymorphisms); trancriptome sequence (quantification of gene expression and alternative splicing, transcript annotation); DNA methylation; species classification by epigenome and

metagenome. However, different research directions still have different advantages and limitations (van Dijk *et al.* 2014).

Table 2.3 Applications of next-generation sequencing technologies

Category	Examples of applications	References
Genome	*de novo* sequencing: the initial generation of large eukaryotic genomes	(Velasco *et al.* 2007) (DiGuistini *et al.* 2009) (Huang *et al.* 2009) (Li *et al.* 2010)
	whole-genome re-sequencing: comprehensive SNP, indels, copy number and structural variations in individual human genomes	(Bentley 2006) (Ossowski *et al.* 2008) (Denver *et al.* 2009) (Xia *et al.* 2009)
	targeted re-sequencing: targeted polymorphism and mutation discovery	(Hodges *et al.* 2007b) (Porreca *et al.* 2007) (Harismendy & Frazer 2009)
Trancriptome	quantification of gene expression and alternative splicing; transcript	(Axtell *et al.* 2006) (Sultan *et al.* 2008)
	annotation; discovery of transcribed SNPs or somatic mutations	(Sugarbaker *et al.* 2008) (Jacquier 2009)
	small RNA profiling	(Berezikov *et al.* 2006) (Houwing *et al.* 2007)
Epigenome	transcription factor with its direct targets	(Johnson *et al.* 2007) (Robertson *et al.* 2007)
	genomic profiles of histone modifications	(Impey *et al.* 2004) (Mikkelsen *et al.* 2007)
	DNA methylation	(Cokus *et al.* 2008) (Costello, Krzywinski & Marra 2009)
	genomic profiles of nucleosome positions	(Fierer *et al.* 2007) (Johnson *et al.* 2006)
Metagenome	environmental	(Edwards *et al.* 2006) (Huber *et al.* 2007)
	human microbiome	(Turnbaugh *et al.* 2007) (Qin *et al.* 2010)

2.2.2 RNA history and types

The central dogma from Francis Crick in 1958 separated DNA and RNA. It supported

the idea that genetic information is transcribed from DNA to RNA and then translated from RNA into protein (Altman & Kirsebom 1999). Jacob and Monod first proposed the concept of mRNA in 1961. They believed that the protein-coding gene is transcribed into an intermediate associated with the ribosome. This intermediate was named as mRNA (Jacob & Monod 1961). After that, Crick thought that the specific amino acid attached to a specific site on the RNA template by the base-pairing principal. Later research identified the existence of and then purified transfer RNA (tRNA) and ribosomal RNA (rRNA) (Altman & Kirsebom 1999; Hoagland *et al.* 1958). The discontinuous distribution of protein-coding sequence (exons) interrupted by non-coding sequence (introns) in the eukaryotic genome means initially introns are part of the mRNA sequence (Chow *et al.* 1977). During the RNA splicing process, alternative splicings are formed so that the introns are cut out from the primary transcripts and degraded; the exons are reassembled into different mature mRNAs (Gilbert 1986). The catalyst function of RNA was detected by Cech in 1970s (Cech 1986). In 1982, Kruger believed that RNA had the dual function of both genetic information storage (like DNA) and catalyst activity (like protein enzymes) (Kruger *et al.* 1982). Afterwards, the RNA interference (RNAi) trait was found. This means double-stranded RNA (dsRNAs) fragments of 21-25 base pair, called small interfering RNA (siRNA), could recognize specific mRNA sequences and then degrade the target mRNAs (Elbashir *et al.* 2001; Fire *et al.* 1998).

Different kinds of RNAs play differential roles in cellular processes. As an overall scheme RNA can be divided into, protein-coding RNAs and non-protein-coding RNAs (ncRNAs). Protein-coding RNAs are named mRNA. ncRNAs means all the RNAs that are not translated into functional proteins. These include house-keeping and regulatory ncRNAs (Eddy 2001). The structural and catalytic roles of house-keeping ncRNAs include tRNAs and rRNAs, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), guide RNAs (gRNAs), RNAs with the function of translation mRNA splicing, rRNA splicing and RNA editing (Altman & Kirsebom 1999).

2.2.3 Transcriptomics

NGS has pushed transcriptomics study into the digital era in the last few years. The first attempt of profiling mammalian transcriptomes was launched in 1991 with the human EST database compiled by a group which applied early automated Sanger sequencing technology. They created a total number of 609 cDNA clones with an average length of

397±99 bases (Adams *et al.* 1991).

The transcriptome, was first described in a scientific paper in1997 and is the full set and quantity of specific transcripts in a cell or tissues. It contains many different transcripts under different developmental or physiological stages (Daniel, Paniz-Mondolfi & Monzon 2015; Marioni *et al.* 2008). The genome variation of the transcriptome arises from use of different tissues or from changes in the physiological or environmental situation. For research on the function of the genome, molecular composition of cells or tissues and biological development and disease, transcriptome research is an essential process. It includes identification of the set of transcripts in a cell, and also their quantity, as they relate to the cell's specific developmental stage or physiological condition (Wang, Gerstein & Snyder 2009). The specific targets of transcriptomics research are all activities involving RNA, which include sequence level and expression level analysis. Analysis includes evaluation of type differences among the RNA's: 2-4% are messager RNA (mRNAs), 80-90% ribosomal RNA (rRNA), 5-15% transport RNA (tRNA), 1% intragenic and intergenic noncoding RNA (ncRNA). There can also be structure differences; start sites, 5' and 3' ends, splicing patterns and post-transcriptional modifications. It is possible to quantify the expression levels of each transcript during development and under different conditions (Costa, Angelini & Ciccodicola 2010).

2.2.4 Early sequence technology of differentially expressed genes

More and more model and non-model organism genomic information has been harvested. Follow the provision of this information functional study of DNA/RNA has become an exciting field of life science research in the post-genome era (Hamilton & Buell 2012). From the gene to the functional mRNA, the cell contains many transcription and post-transcriptional processing activities.

To resolve the wide gap between genome expression and cell function remains challenging. The expression of genetic information from DNA requires that it has to pass through transcription and translation which are finely regulated processes. According to the central dogma of genetics 10 years ago, mRNA is regarded as a bridge to transfer biological information, the first and key regulatory step of gene expression between DNA and proteins (Lockhart & Winzeler 2000). In order to finish these regulation functions, diverse cis-acting proteins combined with gene flanking, "core" and "auxiliary" regions, are necessary. Core elements are extremely strictly required

elements for initiating the pre-mRNA processing; auxiliary elements are very important for promoting or inhibiting the splicing events of genes (Licatalosi & Darnell 2010).

Three kinds of technologies were developed and applied widely to detect differentially expressed genes quickly, which is the early primary goal of transcriptome analysis. They are: 1) hybridization based gene microarray or chip technology, 2) Expressed sequence tag based method (EST, SAGE) and 3) NGS based RNA-sequencing (RNA-seq) technology which depends on short-read pyrosequencing technology of transcriptomics variants (Costa, Angelini & Ciccodicola 2010; Denoeud *et al.* 2008; Luo & Zeng 2001; Morozova, Hirst & Marra 2009; Richmond & Somerville 2000). Among these, SAGE is the earliest method for Digital Gene Expression (DGE) of transcriptomic projects. SAGE made output of the necessarily short reads (18 bp) available when the Solexa machines were first introduced.

Limited by expensive cost and slow throughput, early EST or cDNA library technology, depended on Sanger sequencing. However, it provided genome annotation information in the early days of genome research. Cap analysis of gene expression (CAGE) and serial analysis of gene expression are possible technologies that can be used to achieve transcriptome quantitative analysis (Kocacinar & Sage 2004). However, with EST technology it is difficult to map the short sequences (~20bp) to the genome and it is difficult to afford the high cost of Sanger sequencing based technology. That lead to microarray methods replacing EST in a short time period (Dong & Chen 2013). The theory of the DNA microarray and chip method is based on Sanger sequencing, for which is not necessary to know the genome transcription information. The abundance of RNA is determined by measuring the density of fluorescence of labeled cDNAs. This technology reduces the cost of gene expression profiling. Alternative splicing can be detected by specific microarrays (Clark, Sugnet & Ares 2002). However, a high background of the hybridization methodology is inevitable. Thus it is difficult to distinguish high sequence similarity RNA molecules. In addition, it is also an impossible task for creating a complete genome array as has a high price (Lu & John 2007).

2.2.5 RNA-Seq

In recent years, RNA-Seq has become the most convenient and cost effective tool for quantitative transcriptome profiling. This has been driven by the rapid development of different sequence platforms (Mardis 2008). Studies using RNA-Seq method have

already altered our view of the extent and complexity of eukaryotic transcriptomes (Tang *et al.* 2010). The uses of RNA-Seq have not only included analysis of static genomes, but have also included the dynamic study of transcriptomes (Marguerat & Bähler 2010). For research on the function of the genome, molecular composition of cells or tissues and biological development and disease, transcriptome research is an essential process.

The complications surrounding functions of RNA continues to puzzle investigators because of the many different types of RNA that have been discovered. These include: endogenous small interfering RNA (siRNA), microRNA (miRNA), long interspersed noncoding RNA (lincRNA), promoter- and terminator-associated small RNA (PASR and TASR, resp.), transcription start site-associated RNA (TSSa-RNA), transcription initiation RNA (tiRNA) and others (Chen 2009; Jacquier 2009). However, since transcriptome technology has greatly improved our understanding of the expression of the genome at the transcription level and in terms of gene product function, and genome dynamics. Moreover, this technology can reveal the biological regulation network processes for future plant and crop breeding and stress studies.

For RNA-Seq, total or part of the RNA transcripts are extracted and reverse engineered to cDNAs. After enzymic digestion, millions of billions of short reads of 25–500 bp are massively parallel sequenced using RNA-Seq. Sequence results can be used for qualitative and quantitative study of the transcriptome.

The general RNA-Seq flowchart is described as follows:

After the raw data is harvested by the sequence platform, it undergoes quality control performed by quality control software such as the FastQC tool (http://www.bioinformatics.babraham.ac.uk). Low quality sequence reads (>50% of the bases with a quality score ≤5) and reads containing excess unknown nucleotides (the proportion of N >5%) are always removed.

For samples with a reference genome, the clean sequences reads that have been harvested are mapped to the known reference genome and the software can thus detect the position and expression level of each RNA transcript in the genome (Grabherr *et al.* 2011). Finally, the data goes through a process of confirming the differentially expressed genes across the samples and functional annotation (Hodges *et al.* 2007a).

For samples without a reference genome, *de novo* creation of a reference genome from short reads is necessary. The data are all assembled into contigs, transcripts and unigenes and these are used to detect the position and expression level of each RNA

transcript in the genome (Grabherr *et al.* 2011). In our study, contigs were produced by a default parameter and used *de novo* to generate a non-redundant unigenes set by Trinity software (http://trinityrnaseq.sf.net). Each subclass was a group of all transcripts of a gene. Statistical analysis of each subclass was used to select the longest transcript as the unigene of each subclass and construct a non-redundant unigene set. Finally, the differentially expressed genes were confirmed across the samples (Hodges *et al.* 2007a). By comparing the unigene data with different protein databases, the protein equivalents of unigenes, functional annotation and functional classification are processed. This requires extensive computer resources (Figure 2.2). The *de novo* assembly method has become a hotspot for most of biological transcriptome studies as full genome sequence data has only been created for a few species.

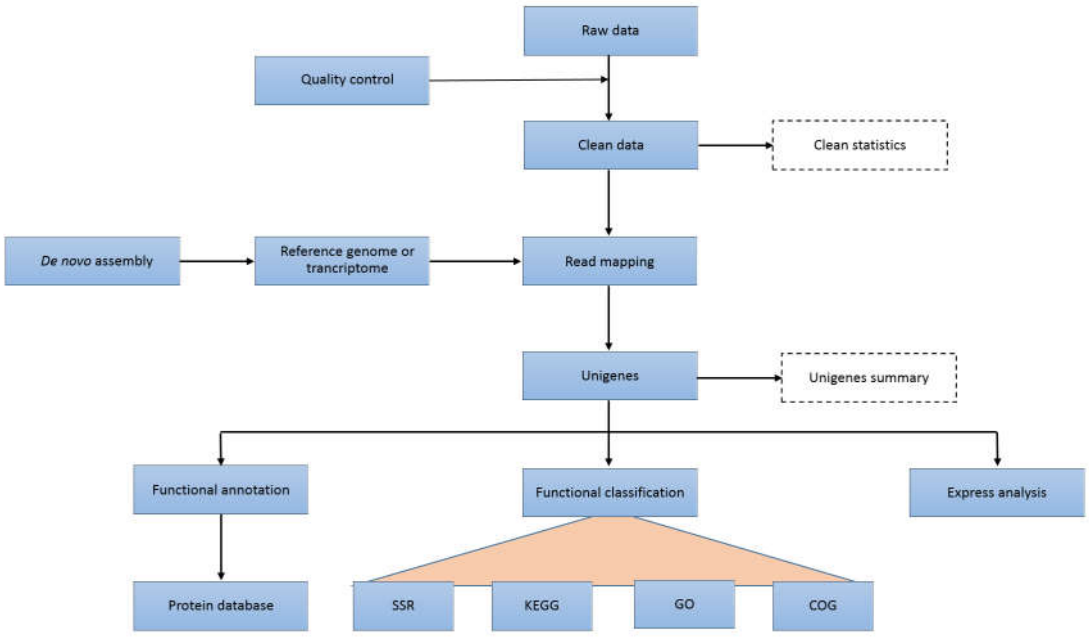


Figure 2.2 RNA-Seq analysis pipeline.

2.2.6 Advantages and disadvantages of RNA-Seq

In recent years, NGS technology has improved RNA study greatly, which has expanded the universe and functional characterization of transcriptomics level studies. It has substantial advantages over previously developed transcriptomic technologies. Although RNA-Seq is still at the preliminary stage of development as a technology. However, some great advantages of this technology have been demonstrated over expression arrays and other powerful tools for transcriptome analysis (Buermans & den Dunnen 2014; Khatoon *et al.* 2014). Compared with expression microarray

technologies, RNA-Seq does not depend on existing genomic sequences, which means characterization of transcription is possible without knowledge of transcription origin in genomic and noncoding RNAs. Expression microarray use is based on the hybridization between gene probes, (which are designed based on existing genome annotation) and microarray and target genes from biological samples (Wang, Gerstein & Snyder 2009). In addition, for RNA-Seq it has been demonstrated that the detection capability can reach to single-base resolution. This results in a greater ability to distinguish RNA isoforms, determine allelic expression, detect sequence variants (for example, SNPs) and reveal the precise location of transcription boundaries (Daniel, Paniz-Mondolfi & Monzon 2015; Marioni *et al.* 2008; Wilhelm *et al.* 2008). In addition, the connection between two exons can be detected by RNA-Seq as it has 30-bp short reads. Meanwhile, longer reads or pair-end short reads could detect connectivity between multiple exons. Both of these are benefits needed to study the complexity of transcriptomes by RNA-Seq (Wang *et al.* 2009). As well low background noise to signal ratio is another one of important advantages of RNA-Seq. This reduces misunderstanding of RNA-seq data, even without known genomic information. The detection ability of differentially expressed genes of RNA-Seq has more advantages as it has a broader dynamic range of expression levels than expression microarray. That is because RNA-Seq relates to the amount of harvested sequences and does not have any quantifiable upper limitation. However, DNA expression microarrays have low sensitivity with very low and very highly expressed genes, which leads to a small dynamic range (Haas & Zody 2010; Wilhelm & Landry 2009). Currently, there are four commercial next-generation sequencing (NGS) platforms available for RNA-Seq: Illumina, SOLID, Ion Torrent, and Roche 454. The different features of them are describe in table 2.4 (Khatoon *et al.* 2014).

Table 2.4 Popular Next-Generation Sequencing Platforms Currently Available for RNA-Seq.

Platform	Mechanism	Read length (bp)	Through put/Run (GB)	Run time	Error rate (%)	Primary errors
Illumina HiSeq 2500	Reversible termination	125	1000	6 days	0.26	Substitution
ABI/LifeTechnology -SOLID 5550 XL	Ligation	120	15	8 days	0.1	A-T bias
ION Torrent 318	H^+ ion sensitive transistor	200	1	2 h	1.71	Insertion/Deletion
Roche 454	Pyrosequencing	400	0.5	10 h	0.8	Insertion/Deletion

The improved quantitative detection ability of RNA-Seq can be used to better characterize both highly produced transcripts and transcripts produced at low levels. This technology also can be used to improve gene annotation, which includes exon/intron boundaries, 5'and 3' and achieved reads mapping to exon junctions defined by GT-AG splicing consensus sites. Both qualitative and quantitative information can be described together in differential alternative splicing (Marioni *et al.* 2008; Pan *et al.* 2008). These advantages have greatly promoted the development of deep sequencing. The main advantages of these technologies are high throughput and savings in money and time costs relative to either tiling arrays or large-scale Sanger EST sequencing (Table 2.5). According to Merioni (2008), RNA-Seq detected 30% more differentially expressed genes than chip inspection in liver and kidney RNA samples studied by the Illumina platform. Moreover, the results have high repeatability and stability for both technical and biological replicates (Marioni *et al.* 2008).

Table 2.5 Advantages of RNA-Seq.

Technology	Tiling microarray	cDNA/EST sequencing	RNA-Seq
		Technology specifications	
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Resolution	Several to 100 bp	Single base	Single base
Throughput	High	Low	High
Reliance on genomic	Yes	No	In some cases

sequence			
Background noise	High	Low	Low
Application			
Simultaneously map transcribed regions and gene expression	Yes	Limited for gene expression	Yes
Dynamic range to quantify gene expression level	Up to a Few-hundredfold	Not practical	>8,000-fold
Ability to distinguish different isoforms	Limited	Yes	Yes
Ability to distinguish allelic expression	Limited	Yes	Yes
Practical issues			
Required amount of RNA	High	High	Low
Cost for mapping transcriptome of large genomes	High	High	Relatively low

2.2.7 Significance of RNA-Seq

Previous RNA-Seq studies have described the content of expressed genomes and complex transcriptomes, consequently these have provided information on regulatory mechanisms. In addition, these studies have also detected extensive post-transcriptional regulation of transcript structures and sequences. RNA-Seq has therefore substantially increased our discoveries and benefitted research and understanding of the transcriptome in the last ten years. With the research moving forward, more and more information will be provided in the future: Firstly, complex sequencing samples will be used to substitute for simple single organism samples, which will provide full transcriptome dynamics information of living communities and interactions within ecosystems (Wang, Gerstein & Snyder 2009). Secondly, RNA-Seq for wild relatives species will help researchers understand the relationships between cultivated and wild species, obtaining information on a diversity of phenotypes and species evolution models and mechanisms will thus be better understood (Marguerat & Bähler 2010). One of the future challenges for RNA-Seq is to explain more complex transcriptomes and the expression changes of rare RNA isoforms from coding mRNA and non-coding mRNA. This will lead to the discovery of novel transcripts and increase our diversity

of knowledge for genomic or epigenetic data. Illumina GA and HiSeq sequencers are the now the dominant sequencing methods in use. In the future, more rapid, cost-effective and powerful evaluation technologies such as Helicos, PacBio and IonTorrent/PGM systems with higher speeds of sequencing and longer sequence fragments will be launched. This will result in increasing research advancing our understanding of RNA and improving the efficiency and accuracy of *de novo* transcriptomics (McGettigan 2013). Advanced technologies will develop more strand-specific sequencing and longer reads to increase coverage and sequencing depth. Thus RNA-Seq depending on more large-scale sequencing will substitute for other sequencing technologies to obtain a greater data harvest.

2.2.8 RNA-Seq research in plants

The biggest advantage of NGS sequencing for genome sequencing is as a technology evolution that has tremendously reduced time and cost requirements for large scale genome sequencing. This is especially so for *de novo* sequencing of eukaryotic genomes, where it provides a more accurate output than other methods (Zhou *et al.* 2010). RNA-Seq plays an important role in molecular biology to understand the activity of hundreds of thousands of genes in parallel, which is a recently developed method to study dynamic transcriptomics by deep sequencing technologies. Gene expression levels under different biological status or different cell types or tissues can be identified by absolute quantification of transcripts (Mutz *et al.* 2013).

The model plant Arabidopsis thaliana was the first de novo plant transcriptome sequenced by the mRNA-Seq method (Weber *et al.* 2007). After quality control, a total of 541,852 expressed sequence tags (ESTs) were harvested by two sequencing projects. The results showed 17,449 genes mapped to the transcriptome. The second sequence project increased the overall sequence coverage by 50%, even if it only increased the number of genes identified by 10%. Mapping of the ESTs to their predicted full-length transcripts indicated that all regions of the transcript were well represented both in transcript length and expression level. More than 16,000 of the ESTs mapped to the genome were not annotated in the known dbEST database. This experiment identified that massively parallel pyrosequencing can be used to improve the annotation of the Arabidopsis genome. Since then, more and more has been done on gene discovery and gene expression of non-model plants which lack complete genomic (Table 2.6). A few recent relevant examples of these studies will be looked at in more detail below to

characterize what types of analyses can be done with the methods available to our group.

Table 2.6 Plant transcriptome sequencing projects in recent years.

Reference Plant	Type of reads	Reference
Arabidopsis thaliana	Roche 454	(Weber *et al.* 2007)
Eucalyptus grandis	Roche 454	(Novaes *et al.* 2008)
Castaneadentata, *C. mollissima*	Roche 454	(Barakat *et al.* 2009)
Olea europaea	Roche 454	(Alagna *et al.* 2009)
Heritiera littoralis, *Rhizophora* mangle	Roche 454	(Dassanayake *et al.* 2009)
Artemisia annua	Roche 454	(Wang *et al.* 2009)
Cucumis sativus	Roche 454	(Guo *et al.* 2010)
Amaranthus uberculatus	Roche 454	(Riggins *et al.* 2010)
Avena barbata	Roche 454	(Swarbreck *et al.* 2011)
Jatropha curcas	Roche 454	(King, Li & Graham 2011)
Cicer arietinum	Roche 454	(Hiremath *et al.* 2011)
Ricinus communis, *Brassica napus*, *Eunonymusalatus*, *Tropaeolummajus*	Roche 454	(Troncoso‐Ponce *et al.* 2011)
Cleome gynandra, *C. spinosa*	Roche 454	(Bräutigam *et al.* 2011)
Triticum aestivum	Roche 454	(Cantu *et al.* 2011)
Cucumis melo (sweetmelon)	Roche 454	(Dai *et al.* 2011)
Pinus sylvestris	Roche 454	(Sun *et al.* 2011)
Pteridium aquilinium	Roche 454	(Der *et al.* 2011)
Pisum sativum	Roche 454	(Furumoto *et al.* 2011)
Utricularia gibba	Roche 454	(Ibarra-Laclette *et al.* 2011)
Phalaenopsis aphrodite	Roche 454	(Su *et al.* 2011)
Triticum aestivum	Roche 454	(Pont *et al.* 2011)
Solanum lycopersicum, *S.habrochaites*	Roche 454	(Bleeker *et al.* 2011)
Eight *Silene* sp. and *Dianthus*	Roche 454	(Blavet *et al.* 2011)
Eucalyptus	Roche 454	(Villar *et al.* 2011)
Lens culinaris	Roche 454	(Kaur *et al.* 2011)
Phaseolus vulgaris	Roche 454	(Kalavacharla *et al.* 2011)
Ziziphus celata	Roche 454	(Edwards, Parchman & Weekley 2011)
Capsicum annuum	Roche 454	(Lu, Cho & Park 2012)
Panicumhallii var.filipes	Roche 454	(Meyer, Logan & Juenger 2012)
Papaver somniferum	Roche 454	(Desgagné-Penix *et al.* 2012)
Scabiosa columbaria	Roche 454 and Illumina	(Angeloni *et al.* 2011)
Cicer arietinum	Roche 454 and Illumina	(Garg *et al.* 2011)
Azadirachta indica	Illumina	(Krishnan *et al.* 2011)

Beta vulgaris	Illumina	(Mutasa-Göttgens *et al.* 2012)
Chorispora bungeana	Illumina	(Zhao *et al.* 2012)
Aegilops variabilis	Illumina	(Xu *et al.* 2012)
Eucalyptusgrandis_E. urophylla	Illumina paired	(Mizrachi *et al.* 2010)
Euphorbia fischeriana	Illumina paired	(Barrero *et al.* 2011)
Hevea brasiliensis	Illumina paired	(Xia *et al.* 2011)
Silene latifolia	Illumina paired	(Chibalina & Filatov 2011)
Taxus marei	Illumina paired	(Ge *et al.* 2011)
Siraitia grosvenorii	Illumina paired	(Tang *et al.* 2011)
Acacia auriculiformis, A. mangium	Illumina paired	(Wong, Cannon & Wickneswari 2011)
Camellia sinensis	Illumina paired	(Shi *et al.* 2011)
Momordica cochinchensis	Illumina paired	(Hyun *et al.* 2012)
Polygonum cuspidatum	Illumina paired	(Hao *et al.* 2012)
Millettia pinnata	Illumina paired	(Huang *et al.* 2012)
Picrorhiza kurrooa	Illumina paired	(Gahlan *et al.* 2012)
Arachis hypogaea	Illumina paired	(Zhang *et al.* 2012)
Agavoideae	Illumina paired	(McKain *et al.* 2012)
Pachycladon fastigiatum, P. cheesemanii	Illumina and Illumina paired end	(Gruenheit *et al.* 2012)
Anthurium andraeanum	Illumina	(Tian *et al.* 2013a)
Zea mays	Solexa/Illumina	(Shan *et al.* 2013)
Vanilla planifolia	Illumina	(Rao *et al.* 2014)
Chrysanthemum nankingense	Illumina	(Ren *et al.* 2014)
Ammopiptanthus mongolicus	Illumina	(Wu *et al.* 2014)
Lilium lancifolium	Illumina	(Wang *et al.* 2014)
Vitis amurensis	Illumina	(Xu *et al.* 2014c)
Tall fescue	Illumina	(Hu *et al.* 2014)
Bean	Illumina	(O'Rourke *et al.* 2014)
Brassica napus	Illumina	(An *et al.* 2014)
Gossypium hirsutum L.	Illumina	(Peng *et al.* 2014)
Trifolium pratense L.	Illumina	(Yates *et al.* 2014)
Haloxylon ammodendron	Illumina	(Long *et al.* 2014)
Asparagus racemosus	Illumina	(Upadhyay *et al.* 2014)
Ocimum	Illumina	(Rastogi *et al.* 2014)
Verticillium dahliae	Illumina	(Salgado *et al.* 2014)
Mangifera indica	Illumina	(Luria *et al.* 2014)
Arachis hypogaea	Illumina	(Chen *et al.* 2014b)
Apium graveolens	Illumina	(Li *et al.* 2014)
Solanum lycopersicum	Illumina	(Spyropoulou, Haring & Schuurink 2014)

white lupin	Illumina	(Secco *et al.* 2014)
Stevia rebaudiana	Illumina	(Chen *et al.* 2014a)
Grapevines barbera	Illumina	(Abbà *et al.* 2014)
Vitis vinifera	Illumina	(Nwafor *et al.* 2014)
Paeonia lactiflora	Illumina	(Zhao *et al.* 2014)
Hordeum spontaneum	Roche 454	(Bedada *et al.* 2014)
Jatropha curcas	Roche 454	(Pan *et al.* 2014)
Japanese cedar	Roche 454	(Mishima *et al.* 2014)
Hevea brasiliensis	Roche 454	(Salgado *et al.* 2014)
Symphonia globulifera	Roche 454	(Brousseau *et al.* 2014)
Virola surinamensis		
Carapa guianensis		
Eperua falcata		
Rubus blackberry	Illumina	(Garcia-Seco *et al.* 2015)
Withania somnifera	Illumina	(Senthil *et al.* 2015)

2.2.8.1 Pathway analysis

Using the next-generation deep sequencing Illumina platform Hiseq2000 technologies Rao *et al*. (2014) studied pathways of vanillin and C-lignin biosynthesis of pods of the vanilla orchid (*Vanilla planifolia*). They did this at different times of development, and for different tissue types, including the seeds, hairs, and placental and mesocarp tissues within the pod. Over 52% (157,191 transcripts) of all 301,459 vanilla transcripts (E-value < 1E-5) obtained were compared with 5 individual reference databases and found to have some homology. 130,550 homologous genes were detected with the NR database. 78,093 genes were found as encoding proteins with conserved domains, while 113,857 genes, 127,510 genes and 14,3894 genes were identified as encoding proteins homologous to proteins in the *Arabidopsis*, rice and sorghum protein databases separately. The analysis found that C-lignin formation in the seed coat involves coordinate expression of monolignol biosynthetic genes with the exception of those encoding the caffeoyl coenzyme A 3-O-methyltransferase for conversion of caffeoyl to feruloyl moieties (Rao *et al.* 2014).

2.2.8.2 Environment effect analysis –cold/freezing/drought

Low temperature during early spring, late autumn, and winter is a primary quality and productivity affecting factor for *chrysanthemum*. Ren *et al*. (2014) generated six separate RNA-Seq libraries (A: cold acclimation (CA) 4°C for one week, B: freezing treatment without prior CA -5°C for 1h and C: freezing treatment without prior CA -

5°C for 2 h, D: freezing treatments with prior CA of 4°C for one week, followed by −5°C for 1 E: freezing treatments with prior CA of 4°C for one week, followed by −5°C for 2 h and F: the control) from leaves and stem. Overall, from library A to library F, 7,270,059, 7,052,023, 7,013,186, 7,228,380, 7,299,665, and 7,468,883 clean reads were obtained respectively. The proportion of clearly mapped reads per library ranged from 77.13% to 81.03% in the libraries. A large number of differential transcribed genes (DTGs) were identified in this study and indicated that more DTGs were identified in the treatments that underwent a prior CA stage than those which had not experienced a CA stage. Moreover, a large amount of unigenes were obtained with KEGG annotation and the major pathways were detected including "metabolism", "biosynthesis of secondary metabolites", "hormone signal transduction", etc. related with cold tolerance. The important detections were confirmed by qPCR also. Finally, the complexity of the regulatory machinery involved in the processes of low temperature acclimation and low temperature/freezing tolerance were confirmed in chrysanthemum (Ren *et al.* 2014).

In *Ammopiptanthus mongolicus* studies, the seedlings were untreated or treated with drought or, cold separately (Wu *et al.* 2014). For *de novo* transcriptome sequencing and assembly, treatments were as follows: Cold was applied as a gradual cooling process under a 16 h dim light/8 h dark cycle. The cooling process started at 4°C and moved to -8°C over a 48 hour period during which time samples were taken. The drought treatments used the same time points (up to 48 h of dehydration) as cold stress. For transcriptome profile analysis seedlings were sampled at 2, 8 and 24 h after the initiation of the cold or drought treatment respectively. 86,058 unigenes were assembled and 51,014 unigenes had an annotated function and 2,440 encoded transcription factors (TFs). Among the results, 2,028 and 2,026 DEGs were common across the three time points of drought and cold treatments respectively, and 971 DEGs were co-regulated by both stresses. 26,999 of the 86,058 unigenes were assigned to 125 KEGG pathways and "Metabolic pathways" was the most important. Moreover, ABA (abscisic acid), BR (brassinosteroid) and ethylene signaling pathways were found to be the main hormone pathways for plants stress response. In addition, the flavonoid biosynthesis genes were enriched in the DEGs co-up-regulated by both stresses and membrane protein genes and genes related to chloroplast were abundant, specifically up-regulated by drought or cold, respectively (Wu *et al.* 2014).

The fresh leaves, stems, bulbs, and roots of *Lilium lancifolium*, an important cold-resistant wild flower for lily cold resistance breeding, were mixed cold treated for 0, 2

h or 16 h at 4 °C (Wang *et al.* 2014). Using RNA-Seq analysis, about 104,703 million clean paired-end reads (90 bp) were obtained from the three libraries. Through use of BLAST analysis with the Swiss-Prot protein database, 18,736 unigenes were obtained. In addition, 25 COG categories were found which formed 12 SOM clusters. The results showed that cold signal transduction genes such as *LlICE* and *LlCDPK* and transcription factor genes such as *LlDREB1/CBF*, *LlAP2/EREBP*, *LlNAC1, LlR2R3-MYB* and *LlBZIP* were expressed highly in the bulb after cold treatment. *LlFAD3, Llβ-amylase, LlP5CS and LlCLS* cold related genes were found to be present with cold treatment and these take part in cellular osmoprotection and carbohydrate metabolism (Wang *et al.* 2014).

Chorispora bungeana, a perennial subnival alpine plant, has strong cold tolerance mechanisms. In order to obtain more information, seedlings were analyzed by Illumina deep-sequencing and compared with *Arabidopsis*. From 7 day old seedlings 10 random plants were selected (roots, shoots and leaves) and subjected to 24 h chilling stress then sampled. After RNA-Seq analysis, a total of 54,870 unigenes were obtained by de novo assembly, with 3,484 chilling up-regulated and 4,571 down-regulated. When *C. bungeana* was compared with *Arabidopsis*, some functional networks of biological processes were the same, such as cold response and molecular functions. The results showed the generally cold related genes *CBF2* and *CBF3* were not found to be up-regulated in *C. bungeana*. In addition, Karrikins were identified as new plant growth regulators involved in chilling responses of both *C. bungeana* and *Arabidopsis*. Moreover, up-regulated genes related to protein phosphorylation and auto-ubiquitination processes were over-represented in both species (Zhao *et al.* 2012).

In *Anthurium andraeanum*, a popular tropical flower, over two billion bases of high-quality sequence and 44,382 unigenes (mean length=560 bp) were produced by Illumina sequencing technology. 27,396 unigenes were functionally annotated (E-value of 10^{-5}). Among the 4,363 identified DGEs, 292, 805 and 708 genes were up-regulated after1-h, 5-h and 24-h cold treatment, respectively. For 1-h cold treatment, the most enriched pathways were: the photosynthesis pathway, metabolic pathways and oxidative phosphorylation pathways. For the 5-h cold treatment, the metabolic pathways and oxidative phosphorylation pathways were significantly up-regulated. After 24-h cold treatment, the mRNA surveillance pathway, RNA transport pathway and plant-pathogen interaction pathway were significantly enriched. In addition, a total

of 39 cold-inducible transcription factors, including subsets of AP2/ERF, Zinc figure, NAC, MYB and bZIP family members were identified (Tian *et al.* 2013b).

2.2.8.3 Maize pathways

Maize (*Zea mays* L.) is a crop planted extensively and used for human consumption and livestock in the world. It is also the most closely related to *Zea mays* ssp. *mexicana* L.*,* the subject of this thesis. High-salinity (150 mM NaCl solution every 4 h), drought (20 % PEG 6000 every 4 h) and cold (4 °C for 24 h) stress responses have been quantified in Maize seedlings by transcriptome analysis (Shan *et al.* 2013). In total, 2,431, 1,448, and 1,264 DEGs were identified in the cold, drought, and salt treatment samples. Among these, 1,176, 558, and 377 genes were up-regulated in different treatment groups and 1,255, 890, and 887 genes were down-regulated, respectively. Six genes were up-regulated in the cold stress treatment but down-regulated in the drought and salt stress treatments. Another 12 genes were down-regulated in cold treatment but up-regulate in the other two stress treatments. Most of the DGEs found in the diterpenoid biosynthesis pathways were involved in the regulation of gibberellin metabolism, such as Gibberellin 2-oxidase (*GA2ox*) genes, gibberellin 3-oxidase (*GA3ox*) genes and gibberellin 20-oxidase (*GA20ox*) genes. Moreover, a number of TFs (DREB, CBF, NAC, bZIP, MYB, and MYC) and functional genes (*LEA, HSP, ROS*) were detected. This study provided more understanding of the genetic basis of the response of maize to different environmental stresses and may be of benefit for improving the abiotic stress tolerance of maize (Shan *et al.* 2013).

2.2.8.4 *Vitis amurensis* pathways

Cold related RNA-Seq analysis has also been used in *Vitis amurensis* studies (Xu *et al.* 2014c). Mixed leaf samples were cold treated for 0, 3, 12, and 48 h. A total of 6,850 cold-regulated transcripts were involved in cold regulation, including 3,676 up-regulated (53.7%) and 3,174 down-regulated (46.3%) transcripts. An important finding was that the total amount of splicing events increased with prolonged cold stress. 38 TF families were detected to be involved in the cold response. In addition, 2,001 DEGs were annotated with KEGG blast for *Arabidopsis* and divided into 113 KEGG pathways (Siriwardana, Kumimoto & Jones 2014). Among these pathways, the most enriched were “Metabolic”, “Biosynthesis of secondary Metabolites”, “Ribosome”, “Plant hormone signal transduction”, and “Plant-pathogen Interaction”. Eight genes were

down-regulated with cold treatment; the ATP synthase delta-subunit gene (*ATPD*), D-ribulose-5-phosphate-3-epimerase (*RPE*), malate dehydrogenase (*MDH*), phosphoribulokinase (*PRK*), lactate/malate dehydrogenase family protein, sulfite reductase , hexokinase-like 1 (*HKL1*), and aspartate aminotransferase 5 (*ASP5*) were exclusively down-regulated. The two predominant up regulated genes were; disproportionating enzymes (*DPE1*) and high cyclic electron flow 1 (*HCEF1*). Some previously uncharacterized *TF* families, which were homologous to *Arabidopsis* counterparts were expressed (*CCAAT_HAP2*, *ABI3VP1*, *ARF*, *PLATZ*, *LIM*, *zf-HD*, atypical_*MYB*, *BBR-BPC*, *C2C2_Zn-Dof*, *C3H*-Type I, *EIL*, *GARP_G2*-like) and some *TFs* attended cold stress response in *V. amurensis, which* provide a genome wide view of the dynamic changes in the transcriptome level (Xu *et al.* 2014b).

2.2.8.5 General pathway summary

As illustrated by the above studies, RNA-Seq analysis technology has been used to detect differential pathways in plants and cereals extensively. For many species which do not have a reference genome, this method has become an effective tool for helping to understand genes expression at the RNA level when exposed to differential biotic and abiotic stresses. Hundreds of thousands of genes have been obtained and received annotation information through BLAST searching with different plant genome databases.

In addition, gene expression profile results have provided numerous examples of up/down regulated genes, which has increased our understanding of response genes and expression levels in differential responses of pathways. In particular, numerous TFs (transcription factors), which have been shown to be the key genes in expression regulation of networks, have been detected. In addition, the changes of differential gene expression at different time stages can provide reference information of gene regulating networks. Some significant related pathways can be annotated and clustered. That annotation provides relationship information of up/down stream genes in the KEGG pathway and describes plant response changes under differential stresses. Moreover, some important gene interaction regulation networks and novel plant regulators can be found and detected.

2.3 Research on Cold response Gene function

2.3.1 Introduction

In order to survive in nature through evolution, plants require effective adaptability and resistance development to adapt to changes in their environment. Through the evolution of genetic type to develop changes in the plant's physiology and shifts in its ecological niche resistance can be development. Low temperature stress is a major environmental factor that deeply impacts on the geographical distribution and species composition of plant communities. It can also lead to a decrease of crop quality and productivity (Puyaubert & Baudouin 2014; Viswanathan & Zhu 2002). The range of cold temperatures that plants may experience can be very large (roughly between 15 and -30 °C in some cold environments). In other more tropical districts, plant species generally survive with only positive temperatures and a narrow range. However, plants in subtropical and tropical areas are influenced by chilling stress even at positive temperatures below 15 to 10 °C. 10 °C is a clear boundary for chilling temperature in most plant species. In addition, some plants show variable susceptibilities to sub-zero temperatures (freezing) (Levitt 1980). The low temperature injury of plants which includes both chilling injury (>0 °C) and freezing injury (<0 °C), restrict the area usable for crop cultivation, and the selection of chilling- or freezing tolerant cultivars has become a major aim for sustainable agriculture in the world (Sanghera *et al.* 2011; Verslues *et al.* 2006).

Signal pathways are a complex mix of biological processes. Thus a full understanding of the mechanisms of plant cold response gene effects has still not been developed and many issues need to be explained. These include characterising how many receptors take part in this process, how they connect with each other and whether other pathways also attend these processes. For cold adaption the cold tolerance related genes, response mechanisms and pathways are comparatively clearer in the model plant *Arabidopsis thaliana*. This is because it is a model plant with a smaller genomic size (125 million bp) which simplifies research and extensively studied (Meinke *et al.* 1998). Therefore, it has contributed substantially towards the understanding the molecular basis of cold tolerance mechanisms. It can offer better reference information for understanding other plants and crops. Many important crops, such as *Zea mays* ssp. *mexicana* L., rice, maize, soybean, cotton and tomato *etc*., are sensitive to chilling temperatures and their ability to withstand freezing and adaptability to low temperature

environmental conditions are poor (Chinnusamy, Zhu & Zhu 2007). However, it is known that there are still some substantial distinguishing differences between the *Zea* genus and the *Arabidopsis thaliana* model plant in system development, morphological physiology and ecological physiology. *Zea mays* ssp. *mexicana* L. can produce different degrees of low temperature damage and the cold response molecular mechanism of *Zea mays* ssp. *mexicana* L. has not been extensively studied.

2.3.2 Cold signalling sensors and messengers

In order to survival in a cold environment, groups of related gene expression timings and levels are reprogrammed and the plant's metabolism is modified. Cold response is a very complex trait involving many different metabolic pathways, gene regulations and cell compartments (Hannah, Heyer & Hincha 2005). Compared to biological stresses for which single genes are often critical, non-biological stress are always affected by multiple genes (quantitative trait). Because of the many genes involved in quantitative traits, there are difficulties in detecting the mechanisms operating with non-biological stresses (Agarwal *et al.* 2006b).

Presently no plants sensors for responding to low temperate have been identified clearly. Most of primary sensors thought to be related with stress sensing appear to be involved. Each sensor may only detect some aspects of the stress not the overall stress in all situations. Different single sensors seem to be involved in the cold signalling pathway (Zhu 2001). Many studies have demonstrated that cell membranes are the primary response location for cold-induced injury (Kratsch & Wise 2000). The theory of membrane lipid phase transformation as a cold signalling mechanism was first proposed by Lyons in 1973. He indicated that low temperature is one of the factors which affects the liquidity of the membrane lipids. Lyons proposed the theory of 'membrane lipid phase transformation', and he thought that the low temperature affects the liquidity of membrane lipid (Lyons 1973). The cold acclimation pathway is regulated and started when plants respond to low temperatures by reducing membrane fluidity and then increasing membrane rigidification. After a cold shock of a few seconds, an influx of Ca^{2+} into the cytosol occurs and the concentration of cytosolic Ca^{2+} is increased in a short time by membrane rigidification to produce IP3, which as a second messenger signal which promotes release of Ca^{2+} from intracellular stores (Knight *et al.* 1991; Ruelland *et al.* 2002). A series of plant *cold responsive* (COR) *genes*, encoding cryoprotective proteins, are transiently increased which can protect

plant cells by avoiding low temperature induced damage (Thomashow 1999).

Generally, the cold stress-induced Ca^{2+} signal can be processed by different pathways. Plants Ca^{2+} sensor groups include CaM (calmodulin) and CMLs (CaM-like), CDPKs (Ca^{2+}-dependent protein kinases), CCaMK (Ca^{2+}-and Ca^{2+}/CaM-dependent protein kinase), CAMTA (CaM-binding transcription activator), CBLs (calcineurin B-like proteins), CIPKs (CBL-interacting protein kinases), SYT1 a homolog of synaptotagmin (Ca^{2+} sensor and initiates exocytosis) and MAP (mitogen-activated protein) (Lissarre *et al.* 2010; Miura & Furumoto 2013). Experiments have demonstrated that CDPKs, calmodulin3 and protein phosphatase 2C play positive or negative regulator functions separately in the regulation of COR gene expression (Tähtiharju & Palva 2001). CBLs deliver the Ca^{2+} signal by interacting with CIPK7 of CIPKs families as identified by the *cbl*1 mutant chilling sensitive phenotype (Huang *et al.* 2011). CAMTA3 has been confirmed as a positive regulator of *CBF2/DREB1C* expression by binding to a regulatory element (CG-1 element, vCGCGb) in its promoter. However, the expression of *CBF3/DREB1A* is not regulated by CAMTA, because the CG-1 element does not exist in its promoter area (Doherty *et al.* 2009). The SYT1 sensor is likely to function in resealing of membranes punctured by ice crystals (Lissarre *et al.* 2010). MKK2 (MAP kinase kinase2) phosphorylates and activates MPK4 and MPK6 in response to cold stress. This has been suggested because the expression level of CBF2/DREB1C [C-repeat (CRT)/dehydration responsive element (DRE) binding protein] was up-regulated in *MKK2* overexpressing plants (Teige *et al.* 2004a).

2.3.3 Cold response related genes

With the development of plant cold tolerance genes studies in recent years, cloning of cold-acclimation genes has become a popular area, which provides numerous candidate genes for developing cold resistant crops by transgenic techniques. Because of the characteristics of non-biological stress, the transfer of a single expressed gene into a crop is not likely to improve cold resistance of a plant substantially. But operation transcription factors can promote the function of multiple genes, which can affect the expression of downstream stress regulated genes and improve the tolerance of a plant (Chinnusamy, Zhu & Zhu 2007). Thus transfer of these genes may have a greater influence on tolerance.

2.3.4 ICE1-CBF/DREB1 dependent pathway of cold signaling

Water and nutrient uptake, membrane fluidity and protein structure are all affected by low temperature. Under cold temperature stress, a plant signal transduction pathway is activated, which includes a series of molecular response mechanisms. Generally, the signal transduction pathway can be divided into different processes, such as cold stress signaling, transcriptional regulation, post-transcriptional regulation, post-translational regulation. Several different sets of cis-acting factors and trans-acting factors are known to depend on abscisic acid (ABA). Others are not dependent on ABA, indicating the involvement of both ABA-dependent and -independent regulatory systems for stress-responsive gene expression (Shinozaki & Yamaguchi-Shinozaki 2000; Thomashow 1999). There are many genes which take part in the cold signaling process, such as receptors, secondary signals (ROS, ABA), second messenger (Ca^{2+}, IP3, cAMP), kinase cascade pathway (MAPK, CDPK), transcription factor (CBF/DREB, bZIP, MYB/MYC), stress response genes (ROS) (Chinnusamy, Zhu & Zhu 2007; Winfield *et al.* 2010). Many key genes take part in plant cold response regulating network (Figure 2.3), such as *ICE, HOS1, MYCR, DREB, CBF/DREB1, DREB2, AREB* and so on.

At present, the best understood important cold acclimation signal regulatory pathway is the ICE1-CBF-COR transcriptional cascade (Chinnusamy, Zhu & Zhu 2007; Garcia-Seco *et al.* 2015; Lissarre *et al.* 2010; Zhu *et al.* 2007). According to *Arabidopsis* microarray studies, 655 up-regulated and 284 down-regulated of the total 24,000 COR genes were detected under cold stress. These influence metabolism, protein stability and cell structure. Most of the highly induced genes are in the CBF pathway. The CBF-dependent pathway is regulated by some important regulators at different gene expression stages, which include transcriptional, post-transcriptional and post-translational levels (Lee, Henderson & Zhu 2005). In this pathway, low temperatures rapidly activate the ICE cold-induced genes, including C-repeat (CRT)-binding factors (CBFs)/dehydration responsive element binding factors (DREBs), which can combine with the promoter area of the *COR* genes and the function of transcription of these genes can be activated (Thomashow 1999).

2.3.5 Transcription factors ICEs controlling cold signaling through the regulation of CBF/DREB1s

The transcription factor *Ice1* mutant plant of *Arabidopsis* is sensitive to both chilling and freezing stresses. Freezing tolerance can be increased by overexpression of this

gene. *Ice1* can bind to the MYC recognition cis-elements (CANNTG) in the promoter of CBF3/DREB1A and induce the expression of CBF3/DREB1A and its regulons during cold acclimation. Nearly 40% of COR genes and 46% of cold-regulated transcription factor genes are regulated by *ICE1*. So it is possible that the *ICE1* gene controls CBF3/DREB1A and many other COR genes (Lee, Henderson & Zhu 2005).

Phosphorylation reactions induced by low temperature were identified in previous studies and these contribute to the process of modification after translation. A small ubiquitin-like modifier (Matsumoto *et al.*) mediated by SIZ1 protein plays an important role in the transcriptional activity of the ICE1 protein (Miura *et al.* 2007). Meanwhile, the ubiquitylation process also takes part in the expression of ICE1. SIZ1, a SUMO E3 ligase, can promote ICE1 protein conjugation with the SUMO compound, which makes modified ICE1 proteins that have transcription activity for *CBF3* and mediate the ICE1 protein entire ubiquitylation 26S proteasome biodegradation pathway. The results have identified that the number 403 serine of the ICE1 amino acid sequence played an important role in the ICE1 degradation process. The stability of ICE1 protein can be improved when the serine was replaced by alanine. Moreover, the induced effect of *CBF3* and down-stream COR genes was showed more dominant with low temperature stress (Miura *et al.* 2011). However, the accurate description of the regulation mechanisms for the activation of ICE1 and for transducing signals from environmental detection and the second messengers to ICE1 are still not clear (Dong *et al.* 2006; Miura *et al.* 2007).

2.3.6 Transcriptional regulation of the CBF pathway

Plants cold tolerance can be improved by expression of *Arabidopsis CBF* genes in different plants as the functionality of the CBF signalling pathway is comparatively well conserved (Miura & Furumoto 2013). The main feature of *CBF* genes is that they play important roles in freezing tolerance and they induced by cold stress rapidly and transiently (between minutes and within 3 h) and then attenuate after a short time. After a cold induction of some hours, the expression level of *CBF* genes are obviously higher than other COR genes (the maximum time being within 24 hours). Yiting Shi (2014) suggested that it is possible that expression level is related to the level of CBF protein accumulation, which induces expression of *COR* genes or that the CBF proteins are modified or have molecular chaperones which are activated more slowly by cold (Shi, Ding & Yang 2015).

In *Arabidopsis*, tomato and rice studies, the function of the *COR* gene cold response has been identified by overexpression of the *CBF/DREB1A* inducer (Gilmour, Fowler & Thomashow 2004; Gilmour *et al.* 2000; Hsieh *et al.* 2002; Ito *et al.* 2006a). CBF genes are affected not only by cold, but are also affected by the circadian clock and light quality signalling in response to environment signals (Dong, Farré & Thomashow 2011; Franklin & Whitelam 2007; Lee *et al.* 2012b). CBF/DREB1s can combine with CRT/DRE cis-elements (A/GCCGAC) of the promoter of *COR* genes to regulate expression of *COR* genes, which belong to the ERF/AP2 (ethylene-responsive element binding factor/APETALA2) type transcription factor family. The CBF/DREB1 (especially CBF3/DREB1A) pathway is controlled by a MYC-type transcription factor ICE1 (Chinnusamy *et al.* 2003). Moreover, *ICE2*, a homolog gene of ICE1 positively regulates CBF1 expression and enhances plant freezing tolerance (Fursova, Pogorelko & Tarasov 2009; Kurbidaeva, Ezhova & Novokreshchenova 2014). A total of three *CBF/DREB1* genes, *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A* have different functions and are possibly organized in a series on chromosome IV in *Arabidopsis* (Medina *et al.* 1999b). In a *Medicago truncatula* study, this kind of tandem array structure of CBF/DREB1 genes was also detected and located in a major freezing tolerance QTL region on *Medicago truncatula* chromosome 6 by QTL mapping using the LR3 population derived from a cross between the freezing-tolerant accession F83005-5 and the freezing-sensitive accession DZA045-5 (Tayeh *et al.* 2013). According to previous *Arabidopsis* transcriptome results, 12% of *COR* genes are controlled by the CBF/DREB1s, with no target specificity among them (Zeller *et al.* 2009). Some transcription factors (ERF/AP2 factors, RAP2.1 and RAP2.6 and the C2H2-type zinc finger, STZ/ZAT10) are regarded as the CBF-regulon (Fowler & Thomashow 2002). CBF/DREB1 homologs have been detected and studied in different species. Most of them have similar functions to the *Arabidopsis CBF/DREB1s* family members. Cold tolerance has been improved by overexpression of different sources of these genes added to genetically modified transgenic rice, tobacco or *Arabidopsis* plants. Cold-regulated CBF-regulon genes have been enhanced also.

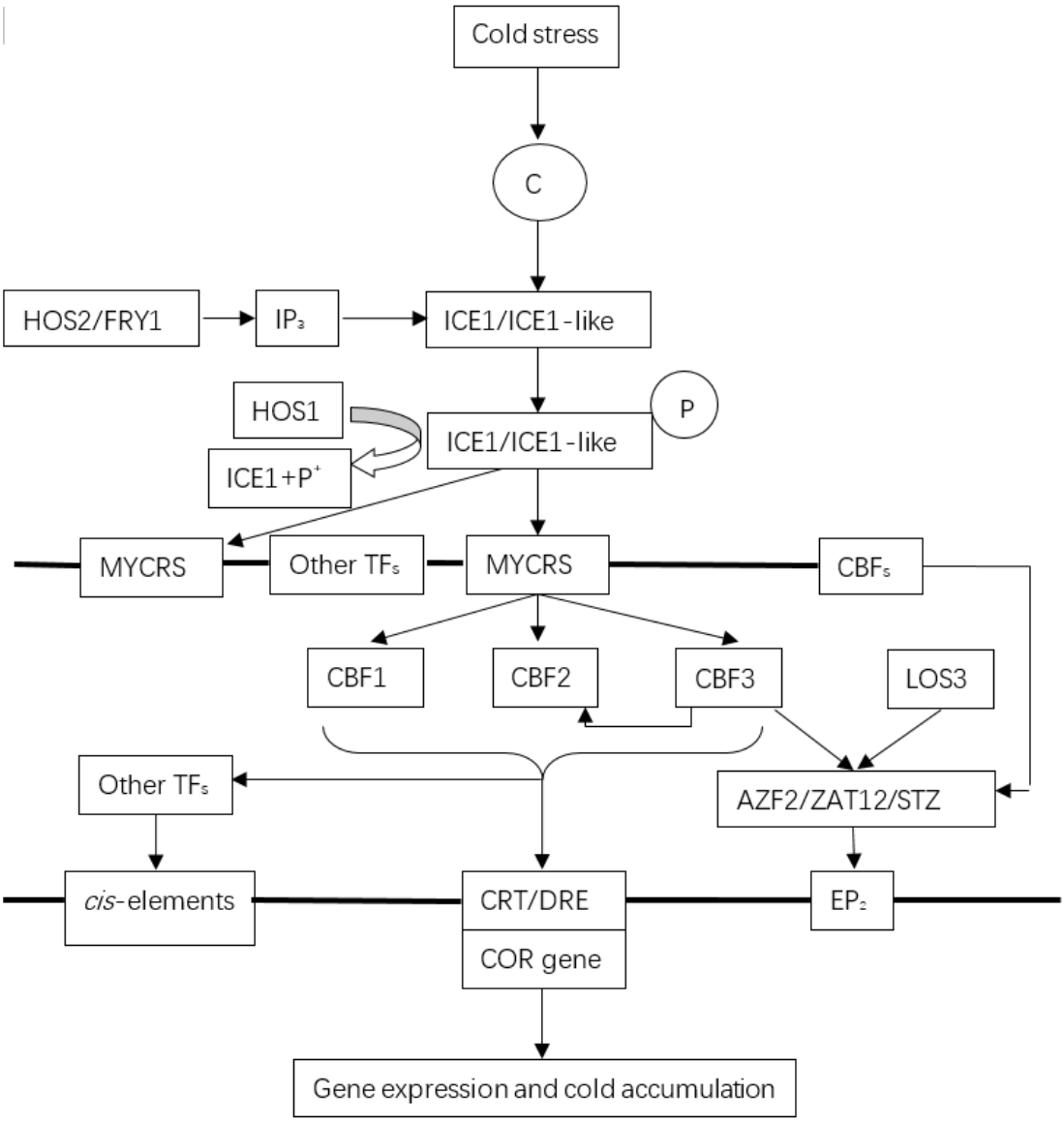
Previously gene expression analysis results showed that, *CBF2/DREB1C* negatively regulates the expression of *CBF1/DREB1B* and *CBF3/DREB1A* during cold acclimation, and *CBF1/DREB1B* and *CBF3/DREB1A* are not involved in regulating other *CBF/DREB1* genes, which have lower cold acclimation ability and play a subsidiary function in the CBF/DREB1 pathway and cold acclimation (Gilmour,

Fowler & Thomashow 2004; Novillo *et al.* 2004; Novillo, Medina & Salinas 2007). However, the *cbf2* mutant plant clearly manifests a freezing tolerance phenotype with or without cold acclimation (Novillo *et al.* 2004). Knockdown of *CBF1* and/or *CBF3* increases plant sensitivity to freezing stress after cold acclimation (Novillo, Medina & Salinas 2007). During the early stages of the cold response, *CBF1/DREB1B* and *CBF2/DREB1C* respond in *ice1* mutation plants, but in the later stages they are not induced. It is suggested that other ICE1-like proteins attend the cold induction of *CBF1/DREB1B* and *CBF2/DREB1C.* This suggestion is verified by the overexpression of *ICE2*, a member of ICE family. It induced the expression of *CBF1/DREB1B* and improved the freezing tolerance in *Arabidopsis* (Fursova, Pogorelko & Tarasov 2009). Compared to *CBF2/DREB1C, CBF1/DREB1B* and *CBF3/DREB1A* both responded at the same time, but *CBF2/DREB1C* lagged behind. Generally, the functions of CBF1/DREB1B and CBF3/DREB1A are different from CBF2/DREB1C. In studies of mangrove *Avicennia marina,* European bilberry (*Vaccinium myrtillus*) and *Brassica rapa* differences in cold responses have been found (Lee *et al.* 2012b; Oakenfull, Baxter & Knight 2013; Peng *et al.* 2013). Because freezing tolerance and expression of *CBF1/DREB1B* and *CBF3/DREB1A* are increased in the *cbf2* mutant, *CBF2/DREB1C* it is possibly a negative regulator of both *CBF1/DREB1B* and *CBF3/DREB1A* (Miura 2013)(Figure 2.3).

CBFs activate some positive transcriptional regulators, such as CAMTA3 (calmodulin-binding transcription activator 3), which can bind to the *CBF2* promoter. CRLK1 (Ca^{2+}-binding calmodulinlike receptor protein kinase) and interacting gene MEKK1 (repressing MAPK kinase activity) (Yang *et al.* 2010). *CAX1* (*CALCIUM EXCHANGER 1*) encodes a vacuolar Ca^{2+}/H^{+} antiporter involved in the regulation of intracellular Ca^{2+} levels. It increased expression of CBF/DREB1 genes and development of the cold-acclimation responses in *Arabidopsis* by induction of CBF/DREB1 and downstream genes (Catalá *et al.* 2003). Negatively regulated transcription factors and some downstream elements from *CBFs* include MYB15 an R2R3-MYB family of transcription factors which can bind to MYB recognition elements in the promoters of CBF genes. Overexpression of MYB15 results in reduced expression of CBF genes and the myb15 mutant plants had increased tolerance to freezing stress (Agarwal *et al.* 2006a). ZAT12 (a C2H2 zinc finger protein) and *EIN3* (a TFs of the ETH signaling pathway), both of them also regulate expression of CBFs. ZAT12 down-regulated the expression of the CBF genes. EIN3 can bind to the

promoters of CBFs and negatively regulates expression of downstream CORs (Davletova *et al.* 2005; Guo & Ecker 2003; Vogel *et al.* 2005). *Hos1* (RING E3 ligase) is an important negative regulator of gene transcription during low temperature. The *Hos1* mutation causes enhanced expression of CBF TFs and downstream CORs genes (Dong *et al.* 2006; Lee *et al.* 2001). *FRY1 encodes an inositol polyphosphate 1-phosphatase.* The mutant plant is a negative regulator in tolerance to freezing, drought, and salt stresses (Xiong *et al.* 2001). In addition, some *CBF* homologous genes were also detected in *Arabidopsis* with different responses and resistance, such as *CBF4 and DDF1*. Among them, *CBF4* is regulated by drought and ABA, but does not respond to cold stress (Haake *et al.* 2002). The *AP2* transcription factor, *DDF1* is up-regulated by low temperatures, salt, drought and heat stresses. By overexpression in wild *Arabidopsis* plants the phonotype showed the involvement of a GA pathway in FTL1/DDF1-mediated tolerance by cross-responses in the pathways (Kang *et al.* 2011).

Furthermore, different types of plant hormones, including abscisic acid (ABA), cytokinins (CKs), jasmonic acid (JA), gibberellins (GAs), brassinosteroids (BRs), ethylene (ETH) and auxin, play important functions in regulating plant freezing tolerance by CBF dependent or -independent pathways (Shi, Ding & Yang 2015).



(Chinnusamy, Zhu & Zhu 2007; Jung *et al.* 2013; Lee *et al.* 2012a; Liu *et al.* 1998; Shinozaki, Yamaguchi-Shinozaki & Seki 2003).

Figure 2.3 Gene expression and the cold accumulation network.

2.3.7 Cold-Regulated Genes

The accumulation of hydrophilic proteins can form an amphipathic a-helix, which is one of the best documented responses of plants to cold treatment (Eriksson *et al.* 2011). Most of these proteins are named as COR (cold responsive), LTI (low temperature induced), RAB (responsive to abscisic acid), KIN (cold induced) or ERD (early responsive to dehydration). These proteins include the dehydrins, which belong to group II of the late embryogenesis abundant (LEA) proteins (Theocharis, Clément & Barka 2012).

COR plays an important role in chilling tolerance and cold acclimation of plant studies. In *Arabidopsis*, group 2 LEA (LEA II) proteins, CORs genes such as *COR78/RD29A, COR47, COR15, COR6.6, COR413* and other dehydrins are induced by cold stress. Among these, LEA proteins are related to membrane stabilization and prevent protein aggregation. Overexpression of COR genes can significantly improve plant cold tolerance. However, the details of regulation and the functions of these COR genes are still not known (Hajela *et al.* 1990; Hundertmark & Hincha 2008; Ingram & Bartels 1996; Shao, Liang & Shao 2005). *RCF1*, which does not play a role in mRNA export, encodes a cold-inducible DEAD (Asp-Glu-Ala-Asp) box RNA helicase for maintaining proper splicing of pre-mRNAs and is important for cold-responsive gene regulation and cold tolerance in plants. *Rcf1-1* mutant plants also identified that some genes are regulators of cold-responsive genes and cold tolerance, such as CIR1 (myb family transcription factor circadian1) and SPFH (spfh/phb domain-containing membrane-associated protein) and these are cold-inducible positive regulator genes; SK12 (shaggy-like serine/threonine kinase12) and PRR5 (pseudo-response regulator5) are cold-inducible negative regulater genes (Guan *et al.* 2013).

Moreover, molecular chaperones HSP (heat shock protein) expression also attends cold response in plants (Santhanagopalan *et al.* 2015). These HSPs, include Hsp100, Hsp90, Hsp70, Hsp60 and small heat-shock proteins (sHsps). Among these Hsp70, sHsp17.6 and Hsp90 (Hsp85 and Hsp87) possibly play a key role in the development of cross adaptation to temperature stress induced by heat acclimation (HA) or cold acclimation (CA) pretreatment in plants (Al-Whaibi 2011). Some PR (pathogen-related) proteins in *Arabidopsis*, such as PR1, PR2 (β-1, 3-glucanase) and PR5 (thaumatin-like proteins), are induced by cold stress (Seo *et al.* 2008). PR10 (Bet v-1 homologues), PR11 (chitinases) and PR14 (lipid transfer proteins) are also cold-inducible in other species (Van Loon, Rep & Pieterse 2006). *STA1* (stabilized 1), an *Arabidopsis* pre-mRNA processing factor 6 homolog, encodes a nuclear protein, which is similar to the human U5 small ribonucleoprotein–associated 102-kD protein and to pre-mRNA splicing factors Prp1p and Prp6p of yeast. *STA1* is required for pre-mRNA splicing and turnover of unstable transcripts of COR genes. *STA1* expression is up-regulated by cold stress, *sta1-1* mutants are defective in the splicing of the cold-induced COR15A and responding to cold stress (Chaabane *et al.* 2012; Lee *et al.* 2006). In addition, many enzymes take part in the differential cold response pathway, such as detoxification and antioxidant cascades, photosynthesis, lignin metabolism, secondary metabolism, cell

wall polysaccharide remodeling, starch metabolism, sterol biosynthesis and oligosaccharide synthesis.

2.3.8 ICE1 gene of different plants

Many different types of ICE1 genes with similarly conserved domain structure have been detected and cloned in different higher plants (Table 2.7). Moreover, many studies have demonstrated that improved low temperature responses of different transgenic plants were successfully induced by adding *ICE1*. In cucumber research, heterologous overexpression of *Arabidopsis thaliana ICE1* has been identified as increasing chilling tolerance and improving the relative physiological indexes of soluble sugars and proline (Liu *et al.* 2010). Transgenic tobacco over-expressing *VaICE1* from *Vitis amurensis*, with its basic helix-loop-helix domain, has a higher chilling tolerance and survival ability under cold stress by improving the activities of superoxide dismutase, peroxidase, and catalase, as well as the chlorophyll yield (Dong *et al.* 2013).

Table 2.7 ICE1-CBF/DREB1-dependent signaling components genetically added to different plants and the resulting cold and other tolerances induced.

Gene	Transgenic host	Source plant	Phenotype and effects	References
AtICE1	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Freezing tolerance; activation of CBF3/DREB1A	(Chinnusamy *et al.* 2003)
AtICE1	*Rice*	*Arabidopsis thaliana*	Cold tolerance	(Xiang *et al.* 2008)
ValICE1 *ValICE2*	*Arabidopsis thaliana*	*Vitis amurnensis*	Freezing tolerance; activation *CBF1*, *COR15A*, and *COR47*	(Xu *et al.* 2014a)
ValICE1	*Tobacco*	*Vitis amurnensis*	Chilling tolerance	(Dong *et al.* 2013)
EcalICE1	*Tobacco*	*Eucalyptus camaldulensis*	Cold tolerance	(Lin *et al.* 2014)
AtICE2	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Freezing tolerance; activation of *CBF1/DREB1B*	(Fursova, Pogorelko & Tarasov 2009)
AtICE1	*Cucumis sativus*	*Arabidopsis thaliana*	Chilling tolerance; dwarf	(Liu *et al.* 2010)

SlICE1	*Solanum lycopersicum*	*Solanum lycopersicum*	Chilling tolerance; accumulation of antioxidants	(Miura *et al.* 2012)
SlICE1a	*Tobacco*	*Tomato*	Cold tolerance; Salt tolerance	(Feng *et al.* 2013)
TaICE141, TaICE187	*Arabidopsis thaliana*	*Triticum aestivum*	Freezing tolerance	(Badawi *et al.* 2008a)
ItICE1	*Oryza sativa*	*Isatis tinctoria*	Cold tolerance	(Xiang *et al.* 2013)
VaICE1	*Tobacco*	*Vitis amurensis*	Chilling tolerance; survival ability and chlorophyll yield	(Dong *et al.* 2013)
AtCBF1, AtCBF2, AtCBF3	*Arabidopsis thaliana*	*Triticum aestivum*	Freezing tolerance	(Gilmour *et al.* 2000; Jaglo-Ottosen *et al.* 1998; Liu *et al.* 1998)
BcCBF1, BcCBF2	*Brassica pekinensis*	*Brassica pekinensis*	Cold tolerance;	(Zhang *et al.* 2006)
OsDREB1A, OsDREB1B, OsDREB1C	*Oryza sativa*	*Oryza sativa*	Chilling, salt and drought tolerance; dwarf	(Ito *et al.* 2006b)
HvCBF4	*Oryza sativa*	*Hordeum vulgare*	Chilling, drought and salt tolerance	(Oh *et al.* 2007)
TaDREB2, TaDREB3	*Triticum aestivum*	*Triticum aestivum*	Freezing and drought tolerance; dwarf	(Morran *et al.* 2011)
AtCBF1, AtCBF2, AtCBF3	*Brassica napus*	*Arabidopsis thaliana*	Freezing tolerance; constitutive expression of *COR*	(Jaglo *et al.* 2001)
AtCBF1	*Fragaria ananassa*	*Arabidopsis thaliana*	Freezing tolerance	(Owens *et al.* 2002)
AtCBF3	*Solanum tuberosum*	*Arabidopsis thaliana*	Freezing tolerance	(Behnam *et al.* 2007)
AtCBF1	*Populus tremula x alba*	*Arabidopsis thaliana*	Freezing tolerance	(Benedict *et al.* 2006)
AtCBF3	*Triticum aestivum*	*Arabidopsis thaliana*	Freezing tolerance	(Pellegrineschi *et al.* 2004)
AtCBF3	*Oat*	*Arabidopsis thaliana*	Salinity stress	(Oraby & Ahmad 2012)

SlCBF1	*Arabidopsis thaliana*	*Solanum lycopersicum*	Freezing tolerance	(Kasuga *et al.* 2004)
AtDREB1A	*Rice*	*Arabidopsis thaliana*	Drought tolerance	(Ravikumar *et al.* 2014)
OsDREB1A	*Arabidopsis thaliana*	*Oryza sativa*	Freezing, drought and salt tolerance	(Zhang *et al.* 2004)
ZmDREB1A	*Arabidopsis thaliana*	*Zea mays*	Freezing and drought tolerance; dwarf	(Dubouzet *et al.* 2003)
VrCBF1, VrCBF4	*Arabidopsis thaliana*	*Vitis riparia*	Freezing and drought tolerance; dwarf	(Qin *et al.* 2004)
HvCBF3	*Arabidopsis thaliana*	*Hordeum vulgare*	Freezing tolerance	(Siddiqua & Nassuth 2011)
LpCBF3	*Arabidopsis thaliana*	*Lolium perenne*	Freezing tolerance; dwarf	(Skinner *et al.* 2005)
SlCBF1	*Arabidopsis thaliana*	*Solanum lycopersicum*	Chilling and oxidative tolerance	(Hsieh *et al.* 2002)
MbDREB1	*Arabidopsis thaliana*	*Malus baccata*	Chilling, drought and salt tolerance	(Yang *et al.* 2011)
GmDREB3	*Arabidopsis thaliana*	*Glycine max*	Freezing, drought and salt tolerance	(Chen *et al.* 2009)
BpCBF1	*Arabidopsis thaliana*	*Betula pendula*	Freezing tolerance; dwarf	(Welling & Palva 2008)
OsDREB1B	*Nicotiana plumbaginifolia*	*Oryza sativa*	Freezing, oxidative and drought tolerance; disease resistance	(Gutha & Reddy 2008)
ZmDREB1A	*Arabidopsis thaliana*	*Zea mays*	Cold tolerance; salt tolerance	(Qin *et al.* 2004)
LcDREB3a	*Arabidopsis thaliana*	*Leymus chinensis*	Drought and salt tolerance	(Peng *et al.* 2011)
CsICE1 *CsCBF1*	*Camellia sinensis*	*Camellia sinensis*	Chilling treatment	(Wang *et al.* 2012)

2.4 Conclusion

Zea mays ssp. *mexicana* L. as one of the closest wild species of cultivated maize, is used to enhance maize genetics and breeding. Compared to another ancestor *Zea mays* ssp. *parviglumis L.* (distributed in the lowlands), *Zea mays* ssp. *mexicana* L. is mainly

distributed in the highlands in Mexico. Both *parviglumis* and *mexicana* have high genetic diversities (Vann, 2015(Sundberg & Orr 1990). The diversified wild gene resources provide enriched selections in target gene modification activities. Previous studies have mainly focused on the teosinte *Branched 1* tilling genes at the molecular level, because the tillering trait is an important agronomic character of the shoot for optimizing plant architecture for maximum yield in cereal product (Clark *et al.* 2006).

The level of molecular biological studies are still not adequate. Only general and fundamental studies have so far been carried out on *Zea mays* ssp. *mexicana* L. The majority of studies refer to the origin, evolution and physiological indexes describe research and cross breeding for using the advantages of F_2 to create hybrid materials (De Wet & Harlan 1976; Fukunaga *et al.* 2005; Wang *et al.* 2008). In the projects described later, we prepared seedling materials of *Zea mays* ssp. *mexicana* L. for RNA-Seq. Then hundreds of thousands of reads were BLAST searched with NCBI nr database to obtain annotations of different reads. Based on this annotation information, we tried to assemble the full CDS coding sequencing of selected genes, which were then BLAST searched with homologous genes of differential species. The results were compared to obtain important information of target genes, including the components of conserved domains, translated protein sequencing information and homology among similar species with known full genome sequencing information (the maize B73) (http://www.maizegdb.org/genome/). After that, we used assembled sequence information to design Polymerase Chain Reaction (PCR) primers and used *Zea mays* ssp. *mexicana* L. extracted genome DNA as a template for target gene clones.

In order to obtain accurate target gene sequencing, single cloned gene products were combined with special T-vector for DNA sequencing. The multiple sequencing results were compared with assembled sequencing of target genes for corrected nucleotide sequencing. Finally, two of the unknown cold response genes were cloned successfully. In addition, the transgenetic system of *Zea mays* ssp. *mexicana* L. has not previously been described. Therefore, one of the important tasks of the lab is constructing transfer genes systems for *Zea mays* ssp. *mexicana* L. However, the current work is to transfer targets genes into *Arabidopsis thaliana* mutant and wild type plants (allogenic transformation) for identifying the cold response effects of these genes by overexpression and complementation methods.

Five wild species of *Zea mays* ssp. *mexicana* L. (K 69-6, W.S.T. 85-2, W.S.T. 92-1, MGB-CI 19, MGB-CI 20) from the International Maize and Wheat Improvement

Center (CIMMYT) have been introduced into the study. They will be used to study ecological and physiological traits in the South China areas. Because most domesticated strains indicate reduced genetic diversity of the introduced species (Gallavotti *et al.* 2004) the extra wild species will provide increased diversity. Some basic parameters will be produced for explaining the level of diversity resulting from this approach.

Overall, my project aims are designed to understand basic physiological index, cold relative genes adaptability and biological information features at the RNA level of *Zea mays* ssp. *mexicana* L.

Chapter 3 RNA-seq analysis of cold and drought responsive transcriptomes of *Zea mays* ssp. *mexicana* L.

Abstract

The annual *Zea mays* ssp. *mexicana* L. is a member of teosinte, a wild relative of the *Zea mays* ssp. *mays* L. This subspecies has strong growth and regeneration ability, high tiller numbers, high protein and lysine content as well as resistance to many fungal diseases, and it can be effectively used in maize improvement. In this study, we reported a *Zea mays* ssp. *mexicana* L. transcriptome by merging data from untreated control (CK), cold (4 ºC) and drought (PEG2000, 20%) treated plant samples. A total of 251,145 transcripts (N50 = 1,269 bp) and 184,280 unigenes (N50 = 923 bp) were predicted, which code for homologs of near 47% of the published maize proteome. Under cold conditions, 2,232 and 817 genes were up-regulated and down-regulated, respectively, while fewer genes were up-regulated (532) and down-regulated (82) under drought stress, indicating that *Zea mays* ssp. *mexicana* L. is more sensitive to the applied cold rather than to the applied drought stresses. Functional enrichment analyses identified many common or specific biological processes and gene sets in response to drought and cold stresses. The ABA dependent pathway, trehalose synthetic pathway and the ICE1-CBF pathway were up-regulated by both stresses. GA associated genes have been shown to differentially regulate the responses to cold in close subspecies in *Zea mays*. These findings and the identified functional genes can provide useful clues for improving abiotic stress tolerance of maize.

Keywords: *Zea mays* ssp. *mexicana* L., Cold tolerance, Drought tolerance, Transcriptome, Differentially expressed genes

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3.1 Introduction

In order to survive in nature through evolution, plants developed multiple strategies to adjust to various abiotic stresses by promoting a series of physiological and metabolic processes such as stomatal closure, repression of cell growth and photosynthesis, and activation of respiration (Mishra, Heyer & Mishra 2014). Stress induced damages are reduced or removed by improving sugar content, increasing antioxidants, taking mechanical action and inducing molecular chaperone chemicals in plants. The gene regulation networks involved are reviewed elsewhere (Zhu 2016).

Low temperature and drought stresses are major environmental factors that impact on the geographical distribution and composition of plant species. They can also lead to a decrease in crop quality and productivity (Viswanathan & Zhu 2002). A number of genes and alternative spliced isoforms responding to these stresses at the transcriptional level have been reported (Leyva *et al.* 1995; Shinozaki, Yamaguchi-Shinozaki & Seki 2003; Thatcher *et al.* 2016), Transcription factors make up of a large group which extensively involve in these processes (Zhu 2016). Among them a class of DREB/CBF transcription factors which bind to the DRE/CRT element (A/GCCGAC) play roles in the regulation of the expression of target genes in response to cold and drought in *Arabidopsis* (Nakashima *et al.* 2009). DREB/CBF-like genes were significantly up-regulated by both stresses of cold and drought (Agarwal *et al.* 2006b). *AaDREB1* from *Adonis amurensis,* as an example, was capable to enhance tolerance to salt, drought, and low temperature in transgenic *Arabidopsis* and rice (Zong *et al.* 2016). Transcription factors including NAC, bZIP, MYB, and MYC also play important roles in cold and drought stresses. Overexpression of *MlNAC5* (*Miscanthus lutarioriparius*), *SlNAC1 (Suaeda liaotungensis* K.*) and VaCBF4 (Vitis amurensis)* enhanced drought and cold stress tolerance of *Arabidopsis*, respectively (Li *et al.* 2013; Yang *et al.* 2015; Zong *et al.* 2016). MAPK related genes that are involved in signal transduction are significantly induced by both cold and drought (Pitzschke, Schikora & Hirt 2009). A MAPK gene (GRMZM2G174170_T01) from maize, for example,

was activated under cold and drought stresses and was suggested to contribute to stress tolerance in maize (Shan *et al.* 2013). The expression levels of most *ZmVQ* genes (41 out of 61 members) encoding VQ motif-containing proteins were changed by the drought stress, and half of *ZmVQ* genes were co-expressed with *ZmWRKY* genes in maize (Song *et al.* 2015).

Phytohormones such as abscisic acid (ABA), ethylene, cytokinin (CK), auxin (IAA), gibberellin (GA) and jasmonate (JA) play important roles in regulating plant growth and development and also in the responses to various biotic and abiotic stresses (Peleg & Blumwald 2011). ABA synthesis is one of the fastest responses of plants to abiotic stress, triggering ABA-inducible gene expression and causing stomatal closure, thereby reducing water loss via transpiration and eventually restricting cellular growth. 9-cis-epoxycarotenoid dioxygenase (NCED) is a key enzyme in the ABA biosynthetic pathway in several plants which cleaves carotenoids to form the phytohormone ABA (Riahi *et al.* 2013). Cold or drought stress can induce ABA biosynthesis and exogenous ABA can improve cold or drought tolerance in tomato, *Arabidopsis* and tobacco (Thompson *et al.* 2000; Wan & Li 2006; Zhang *et al.* 2008). In maize, thousands of genes are thought to be involved in abiotic stress. In particular, the gibberellin (GA) metabolic genes. Thus understanding their expression changes could help to detect molecular mechanisms of the GA pathway under stress conditions (Colebrook *et al.* 2014). The response of GA metabolic genes to abiotic stress has been investigated and these genes participate in the CBF1-mediated stress-response pathway (Niu *et al.* 2014) and the GA20ox gene that is responsible for GA biosynthesis is down-regulated by cold treatment in *Zea maize* (Shan *et al.* 2013).

Zea mays ssp. *mexicana* L. is a member of teosintes and a close wild relative of cultivated maize (Almeida *et al.* 2011). Although teosintes have not yet been widely used in maize breeding, the high genetic diversity shows that *Zea mays* ssp. *mexicana* L. is a genetic reservoir for the improvement of agronomic characteristics of cultivated maize and

teosintes (Wang *et al.* 2008). Since its introduction to China from Japan in 1979, the planting areas of teosinte have increased extensively in southern China, including Guangdong, Fujian, Guangxi and Sichuan Provinces (Song *et al.* 2005). As an important forage or silage source, teosinte has a larger plant biomass, higher tiller number and better resistances to various stresses than cultivated maize (Niazi *et al.* 2015b). Similar to maize, most lines of *Zea mays* ssp. *mexicana* L. originate from high altitude in northern and central Mexico and can adapt to acid soil with the best growing temperatures ranging from 25 °C to 35 °C (Fukunaga *et al.* 2005). It is considered to be a very cold-sensitive crop, especially during the germination and early autotrophic growth stages, despite it having originated at altitudes of about 1,000-2,000 m (Hincha & Zuther 2014).

RNA-seq (RNA sequencing) has become the most convenient and cost effective tool for understanding gene structure and quantitative transcriptome profiling. It does not depend on the existence of known genomic sequences, and shows a great ability for detecting differentially expressed genes with a broader dynamic range of expression levels (Wang, Gerstein & Snyder 2009). To discover genetic bases and molecular mechanisms in response to cold or drought in *Zea mays* ssp. *mexicana* L., the RNA-seq technique was employed in this study and the transcriptome of *Zea mays* ssp. *mexicana* L. was presented for the first time. In total, 414,232,462 high quality clean reads were obtained by RNA-seq and were used for *de novo* assembly and annotation of genes from *Zea mays* ssp. *mexicana* L. Differentially expressed genes (DEGs) under cold or drought were also identified. These data will be valuable for the exploration of genetic and molecular mechanisms in response to stresses in *Zea mays* ssp. *mexicana* L. and provide gene resources for breeding programs.

3.2 Materials and methods

3.2.1 Plant materials and growth conditions

Plump seeds of *Zea mays* ssp. *mexicana* L. variety “8493” were used in this study. Seeds were washed 3 times with distilled water, then soaked in 75% ethanol and 2% sodium hypochlorite for 10 min and 3 min, respectively, and washed 3 times with distilled water

before planting in plastic boxes (54×28×7 cm) containing soil substrates (Jiffy, Netherlands, http://www.jiffygroup.com/en/substrates/). The boxes were transferred into a climate control box (RXZ 500-C, JIANGNAN Instrument), allowing the seeds to germinate and grow under a photoperiod of 10-h light/14-h dark and a humidity of 60% at 25 ºC. Thirteen-day-old seedlings were assigned into 3 groups for different treatments. For cold treatment (cold), seedlings were treated for 12 h at 4 ºC under 10-h light/14-h dark while for drought treatment (drought), seedlings were treated with Hoagland solution containing PEG2000 (20%) for 3 h under the same light condition. Seedlings of the control group (CK) grew under the same photoperiod and were assigned neither of the above treatments. After treatments, fresh tissues (roots, stems and leaves) were sampled and pooled, followed by quick-freezing with liquid nitrogen and storing at -80ºC for further analysis. Two replicates for each treatment were applied and plant materials from at least three seedlings were pooled for each sample.

3.2.2 RNA extraction

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. RNA was treated with RNase-free DNase I (Takara, Japan) to remove any possible DNA. The integrity was checked by gel electrophoresis and the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Concentration of total RNA was determined using NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE). Total RNA with RIN values ≥7.3 and 28S:18S ratios ≥1.2 were accepted for the following RNA-seq analysis.

3.2.3 RNA-seq analysis

The Illumina HiSeq2500 platform was applied for RNA-seq analysis. To obtain a comprehensive overview of the *Zea mays* ssp. *mexicana* L. transcriptome and the transcript profiles under cold and drought, six libraries were constructed and paired-end sequencing was carried out according to the manufacturer’s instructions (Illumina, San Diego, CA). In

brief, poly(A)-tailed mRNA was enriched using biotin-Oligo (dT) magnetic beads and fragmented into short fragments of 200-700 bp, followed by converting into double-stranded cDNA. The cDNA fragments were then purified with a QiaQuick PCR extraction kit and adapters were added to both ends of the short fragments. cDNA fragment pools were loaded to Illumina HiSeq2500 platform for sequencing. The generated raw data were processed for further analysis. Library construction and RNA-seq were carried out by Novogene Bioinformatics Technology Co. Ltd, China.

3.2.4 *De novo* assembly and sequence clustering

The quality of raw data was controlled by using the FastQC tool (http://www.bioinformatics.babraham.ac.uk). Reads containing substantial unknown nucleotides (the proportion of N >5%) and low-quality reads (>50% of the bases with a quality score ≤5) were discarded, and the first 10 bases of processed data were removed. The generated clean data from each sample were merged to perform *de novo* assembly using Trinity with default parameters and an optimized k-mer length of 25. A set of transcripts were generated and served as a reference transcriptome. Based on the shared sequence, transcripts from a gene were clustered into a subclass and the longest transcript was regarded as the unigene. The above processes were performed by Trinity software with default parameters (http://trinityrnaseq.sf.net, updated on 2014/07/17) (Haas *et al.* 2013). To identify possible plant transcription factors (TFs), transcriptional regulators (TRs) and protein kinases (PKs), Plant Transcription factor & Protein Kinase Identifier and Classifier (iTAK) V1.5 was employed to analyse unigenes with the best match result.

Raw Illumina sequences and assembled sequences are available in the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) (accession number: GSE76939).

3.2.5 Identification of differentially expressed unigenes (DEGs)

The paired-reads from each sample were mapped to an assembled reference transcriptome

by Bowtie software v1.1.1 with default parameters, and the number of mapped reads were calculated by RSEM (v 2.15) (Langmead *et al.* 2009; Li & Dewey 2011). FPKM values were assigned to each unigene, representing normalized expression level by eliminating the influences from library construction and the length of genes (Trapnell *et al.* 2010). Unigenes with FPKM ≥ 1 were used for further analysis. Fold changes for each unigene under cold or drought condition were determined by comparing the FPKM value to that in control sample, and those unigenes with more than 2 fold change and adjusted *P* value <0.005 were identified as differentially expressed genes (DEGs) by DESeq 2 (V 1.2.10) package of the R statistical program (Love, Huber & Anders 2014).

3.2.6 Annotation and function classification of transcripts and unigenes

Annotations were assigned to each unigene based on the top hit in BLASTX search against the protein databases, with the non-redundant (Nr) protein database at GeneBank (http://www.ncbi.nlm.nih.gov) as the highest priority, followed by Swiss-Prot (http://www.expasy.ch/sprot), KEGG (http://www.genome.jp/kegg) and eukaryotic KOG (http://www.ncbi.nlm.nih.gov/KOG) in that priority order. The significant thresholds of E-value were set at $\leq 10^{-6}$. CDS (5'->3') predictions of unigenes were confirmed with the priority result of BLAST searched protein.

The accession number and the GI code of the top hit at the BLASTX search against the Nr database were retrieved and ID mapping was performed to obtain GO annotations for the queried unigenes. To further annotate the unigenes, the Blast2GO (v2.5.0) program was employed to get GO annotations based on molecular function (MF), biological process (BP) and cellular component (CC) features (Conesa *et al.* 2005). GO enrichment analysis were carried out by AgriGO software with FDR<0.05.

3.2.7 Quantitative real-time PCR analysis

To further verify the expression profiles of genes in our Illumina RNA-seq data, total RNAs were extracted from three independent groups, which were different from those used for

RNA-Seq. First-strand cDNA synthesis and qRT-PCR were carried out with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa) and TransStart® Tip Green qPCR SuperMix (Transgen), respectively. qRT-PCR was carried out in the BIO-RAD CFX96 sequence detection system according to the manufacturer's instructions. A total of 12 genes were randomly selected and gene-specific primers were designed with online Primer-blast of NCBI. The unigene annotated maize actin-2 was used as the endogenous control (GeneBank accession number NP_001146931.1). Each PCR reaction (20 μL) contained 10 μL 2× Green qPCR SuperMix, 0.2 μM of each primer and appropriately diluted cDNA. The thermal cycling conditions were 94 °C for 30 s, followed by 40 cycles of 5 s at 94 °C and 30 s at 60 °C then 72 °C for 30 s. At the second dissociation stage, 95 °C for 10 s, 65 °C to 95 °C with increment of 0.5 °C for 0.05 s were used. All reactions were performed in triplicate, including the non-template controls. The relative expression level was calculated with the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

3.3 Results

3.3.1 De novo assembly and functional annotation of the *Zea mays* ssp. *mexicana* L. transcriptome

To comprehensively understand the transcript profiles of *Zea mays* ssp. *mexicana* L. under cold and drought, a total of six samples, including two biological repeats for each condition, were collected and two replicated runs of Illumine sequencing were performed for each sample. Raw reads of 153,219,582, 156,576,828 and 150,941,988 were generated from the control, cold and drought group, respectively. After quality check, the adapters, low-quality sequences and ambiguous reads were removed. A total of 136,233,336 (control), 141,732,126 (cold) and 136,267,000 (drought) clean paired-end reads, corresponding to 17.04 Gb (control), 17.72Gb (cold) and 17.02 Gb (drought) clean bases, respectively, were used for future assembly. *De novo* assembly was carried out using the Trinity software and 251,145 transcripts and 184,280 unigenes with N50 values of 1,269 bp and 923 bp were obtained, respectively (Table 3.1). Approximately 47% of assembled transcripts and 35%

of unigenes had a length of more than 500 bp (Supplemental file 1).

Table 3.1 Overview of the sequencing and assembly of *Zea mays* ssp. *mexicana* L.

Item	Reads	Transcripts	Unigenes
Total number of raw reads	460,738,398		
Total number of clean reads	414,232,462		
Total clean nucleotides (bp)	51,779,057,750		
Average Q20 (%)	93.99		
Contig N50 length (bp)		1,269	923
Maximum sequence length (bp)		17,701	17,701
Average length (bp)		776	631
Total number of transcripts/unigenes		251,145	184,280

Annotations were assigned to each transcript and unigene by BlastX search against the plant protein collections of four public databases (NR, Swiss-Prot, KEGG and KOG). There were124,297 (49.49%) transcripts and 68,177 (36.7%) unigenes that had at least one significant match (E-value<1e-6) in one of these databases. Among all annotated transcripts/unigenes, more than 98.5% had at least one hit in the NR database at NCBI, which allowed retrieving GO annotations by the ID mapping method. A total of 28,382 (15.40%) unigenes were assigned to at least one GO term, among which 9,769; 5,404 and 13,209 unigenes were included into groups of “biological process”, “cellular component” and “molecular function”, respectively (Figure 3.1 Functional classification of assembled unigenes.). Among the “biological process” group, 9.96%, 7.38% and 7.04% were annotated into “fatty acid biosynthetic process”, “pinoresinol biosynthetic process” and “(1->3)-beta-D-glucan biosynthetic process”, respectively. In the “molecular function” group the subgroups of “nucleic acid binding” (9.68%), “RNA-directed DNA polymerase activity” (8.4%) and “zinc ion binding” (7.19%) ranked the highest. Additionally, 15.30%, 7.49% and 7.48% of unigenes were annotated as cellular components of “integral component of membrane”, “cytoplasm” and “ribosome”, respectively (Supplemental file

2).

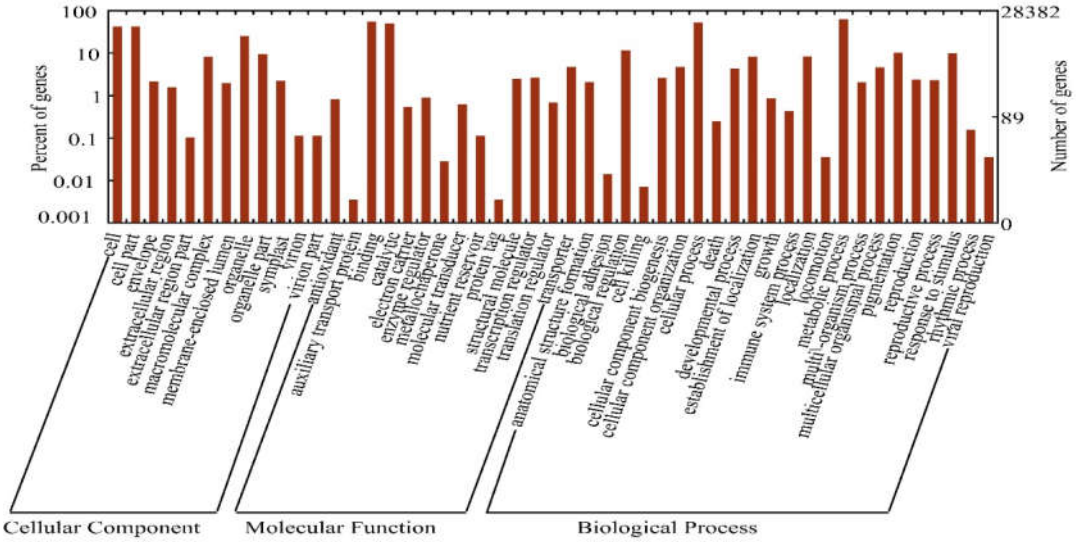

Figure 3.1 Functional classification of assembled unigenes. Functional classification of the assembled unigenes based on Gene Ontology (GO) categorisation.

*GO annotations were assigned to unigenes based on the best BLAST hits, and a total of 98,947 GO terms were retrieved for 28,382 unigenes which were grouped into 49 categories. The left Y-axis represents the percentages of unigenes in each main category. The right Y-axis indicates the numbers of unigenes in each GO category.

Homologs were also identified by searching against the protein database of the most closed relative *Zea mays* L. and the model species, *Arabidopsis thaliana*. A total of 60,583 and 41,293 unigenes had at least one hit sequence from maize and *Arabidopsis thaliana*, respectively, with an E value < 1E-6. After removing the replicate records, 27,379 and 15,643 homologs were obtained, representing 46.78% and 44.47% of the maize and *Arabidopsis* proteome, respectively (Supplemental file 3).

A total of 2,157 and 512 unigenes were identified as transcription factors (TF) or transcription regulators (TR) based on the Blast search against the Nr protein database at NCBI, covering 62 TF families and 25 TR families, respectively (Table 3.2). In addition, predicted protein kinases (PK) that play crucial roles during signal transduction and other

biological processes were also obtained, including 1,598 unigenes covering 67 PK families (Table 3.3).

Table 3.2 Transcription factor (TF) and transcription regulator (TR) gene families identified from *Zea mays* ssp. *mexicana* L.

TF Family	Number	TF Family	Number	TR Family	Number
C2H2	253	MIKC	12	SNF2	77
bZIP	141	ARF	11	GNAT	52
MYB-related	129	C2C2-YABBY	11	PHD	51
bHLH	125	CPP	11	AUX/IAA	51
zn-clus	123	NF-YB	11	TRAF	47
C3H	112	RWP-RK	11	SET	45
Orphans	109	C2C2-LSD	10	HMG	30
ERF	108	E2F-DP	10	mTERF	26
WRKY	93	PLATZ	10	Jumonji	22
NAC	92	SRS	10	SWI/SNF-BAF60b	18
GRAS	77	Alfin-like	9	IWS1	16
HB	64	BES1	9	LIM	15
C2C2-GATA	53	EIL	9	SWI/SNF-SWI3	13
FAR1	48	AP2	5	Rcd1-like	8
G2-like	44	ARR-B	5	MBF1	8
B3	40	CSD	5	ARID	6
HSF	37	DBP	4	LUG	5
MYB	34	BBR-BPC	3	TAZ	5
C2C2-Dof	33	CAMTA	3	DDT	4
LOB	31	GRF	3	SOH1	3
OFP	29	NF-X1	3	Coactivator p15	3
M-type	28	VOZ	3	MED6	2
SBP	26	C2C2-CO-like	2	MED7	2
TCP	24	LFY	2	RB	2
Trihelix	24	Whirly	2	Pseudo ARR-B	1
TUB	23	DBB	1	ULT	1
NF-YA	18	HRT	1	GeBP	12
Tify	18	RAV	1		
NF-YC	15	S1Fa-like	1		
zf-HD	14	STAT	1		

Table 3.3 Protein kinase (PK) gene families identified from the *Zea mays* ssp. *mexicana* L.

PK Family	Number	PK Family	Number
SNF1 Related Protein Kinase (SnRK)	132	Leucine Rich Repeat Receptor VIII	11
IRE/NPH/PI dependent/S6 Kinase	104	Leucine Rich Repeat Kinase VII	11
Leucine Rich Repeat Kinase XI & XII	101	Putative protein kinase/Putative receptor-like protein kinase	10
Domain of Unknown Function 26 (DUF26) Kinase	95	APG1 Like Kinase	8
S Domain Kinase (Type 2)	77	Other Kinase	8
Legume Lectin Domain Kinase	76	Receptor Like Cytoplasmic Kinase IV	8
Receptor Like Cytoplasmic Kinase VII	72	Phosphoenolpyruvate Carboxylase Kinase	7
CDC2 Like Kinase Family	71	Putative receptor like protein kinase	7
Unknown Function Kinase	71	Leucine Rich Repeat Kinase X	7
Calcium Dependent Protein Kinase	63	LRK10 Like Kinase (Type 1)	7
MAPK Family	53	Leucine Rich Repeat Kinase IV	7
GmPK6/AtMRK1 Family	36	Receptor Like Cytoplasmic Kinase II	7
Wall Associated Kinase-like Kinase	36	Leucine Rich Repeat Receptor Kinase I & Unknown Receptor Kinase I	6
Casein Kinase I Family	33	Receptor Like Cytoplasmic Kinase I	6
MAP3K	33	Leucine Rich Repeat Kinase I	6
Leucine Rich Repeat Kinase III	31	WNK like kinase - with no lysine kinase	5
MAP2K	29	Possible MAP2K	4
Leucine Rich Repeat Kinase II & X	28	ATN1 Like Family	4
STE20-PAK Like Protein Kinase	26	Ankyrin Repeat Domain Kinase	4
CRPK1 Like Kinase (Types 1 and 2)	25	Leucine Rich Repeat Kinase IX	4
GSK3/Shaggy Like Protein Kinase Family	24	Tousled like kinase	3

Receptor Like Cytoplasmic Kinase VIII	23	RKF3 Like Kinase	3
Receptor Like Cytoplasmic Kinase IX	20	Wall Associated Kinase	3
Plant External Response Like Kinase	20	Leucine Rich Repeat Kinase VI	3
LAMMER Kinase Family	19	Putative protein kinase/Ser_thr kinase like protein/Putative receptor-like protein kinase	3
S Domain Kinase (Type 1)	17	Light Sensor Kinase	2
Receptor Like Cytoplasmic Kinase VI	17	Calcium/Calmodulin Dependent Protein Kinase (CCamK)	2
Receptor Like Cytoplasmic Kinase V	16	Receptor like protein kinase/Receptor lectin kinase like protein	1
Other Protein Kinase	16	Putative LRR receptor-like protein kinase/Receptor protein kinase like protein	1
Crinkly 4 Like Kinase	16	ELM1/PAK1/TOS3 Like Kinase	1
CTR1/EDR1 Kinase	15	C-terminal Ankyrin Repeat Domain Kinase	1
Receptor-like protein kinase	15	Male grem cell-associated kinase (mak)	1
Leucine-rich transmembrane protein kinase/Strubbelig Receptor Family 1	14	C-terminal Ankyrin Repeat Domain Kinase	1
Casein Kinase II Family	12		

3.3.2 Expression quantification of unigenes and identification of differentially expressed genes (DEGs)

The expression levels of unigenes were evaluated using FPKM values. A total of 43,432 (23.57%), 42,849 (23.25%), 55,928 (30.35%), 48,945 (26.56%), 47,327 (25.68%) and 43,879 (23.81%) unigenes had FPKM ≥1 from the sample of control-1, control-2, cold-1, cold-2, drought-1 and drought-2, respectively. However, the percent of unigenes with FPKM ≥100 from each sample was less than 1%, ranging from 0.78% to 0.84% (Supplemental file 1). Compared with the control, the cold treatment at 4 °C resulted in a

larger number of genes with high FPKM values than did the drought treatment with PEG, indicating that the responses induced by cold was more dramatic than that by drought stress in *Zea mays* ssp. *mexicana* L. at least with the relative levels of stress used in this trial.

To investigate differential responses of *Zea mays* ssp. *mexicana* L. to cold or drought stress, data from replicated treatment groups were merged to perform pair-wise comparisons, and unigenes that matched the criteria of $|\log_2\text{Change}|>=2$ and adjusted *p*-value < 0.005 were identified as differentially expressed genes (DEGs). Totally, 5,338 DEGs were obtained, with 3,049 and 614 genes differentially responding to cold and drought stresses, respectively. Among those cold-responsive DEGs, 2,232 were up-regulated and 817 were down-regulated (Figure 3.2C, Supplemental file 4). Notably, annotations for 41.1% of the up-regulated and 25.7% of the down-regulated DEGs were not retrieved from the public maize database (Supplemental file 4), suggesting some special regulation paths in *Zea mays* ssp. *mexicana* L. For those DEGs which responded to drought, however, the number of DEGs was less, with only 532 and 82 genes being activated and inhibited by drought, respectively, of which 14.47% (77) and 4.2% (17) were unannotated (Supplemental file 4). The expression level of the whole set of DEGs in all six samples were also evaluated by plotting the Log_{10}-transformed FPKM values in descending order (Figure 3.2A), and the distributions of DEGs' expression suggested activation was present in both cold- and drought-treated samples, with more dramatic transcriptional changes in the cold-treated samples. Taken together, these results indicated that *Zea mays* ssp. *mexicana* L. had distinct gene networks in response to cold and drought.

Venn analysis indicated that 194 unigenes were regulated by both factors and 2,855 and 420 were specifically induced by either cold or drought (Figure 3.2B, Supplemental file 4) .Of the 194 shared DEGs, 179 genes had similar response patterns to both cold and drought, including 152 up-regulated genes and 27 down-regulated genes. Two unigenes (c52482_g3, c52482_g6) similar to protein phosphatase 2C3 and an unannotated unigene (c50928_g2) gave the greatest up-regulation, while the c29390_g1 (thioredoxin H4),

c63024_g4 (hypothetical protein ZEAMMB73_373831) and an unannotated unigene (c55190_g2) comprised were the three most down-regulated. Fifteen genes showed the opposite response patterns under cold and drought stresses with 13 genes being down-regulated by cold but up-regulated by drought.

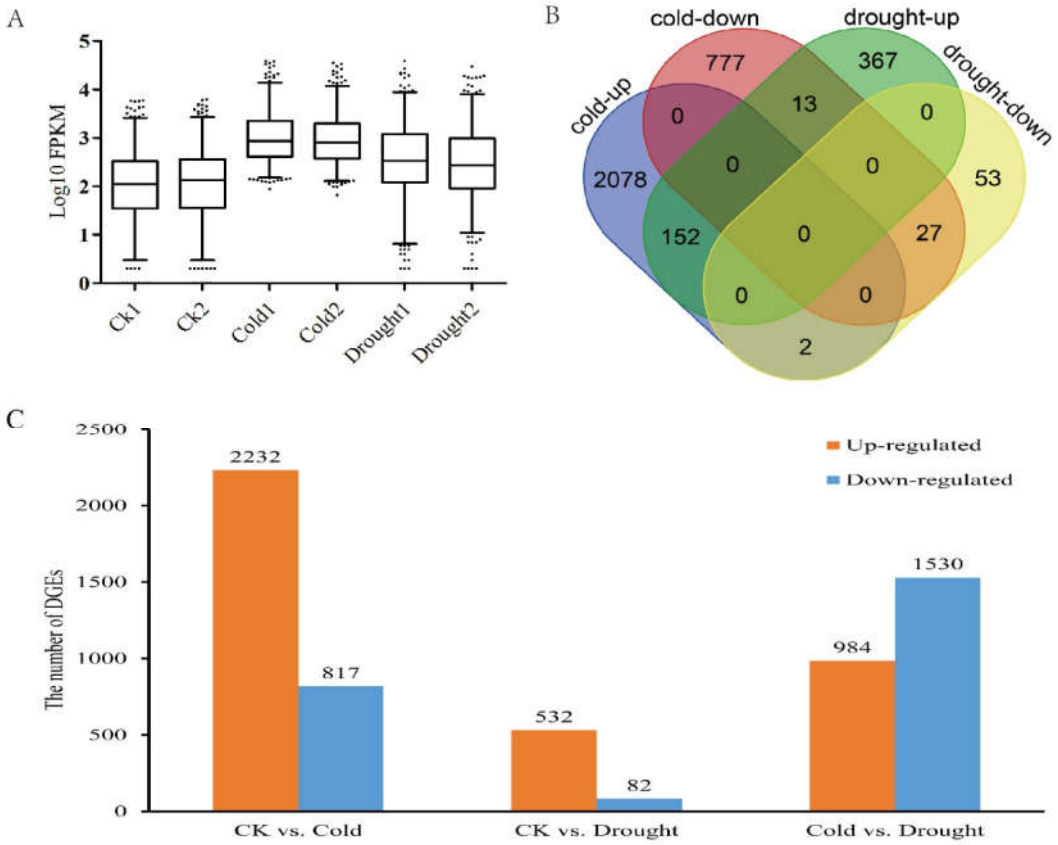

Figure 3.2 Summary gene expressions at the global level of transcriptome.

*(A) Distribution of Log10FPKM of DEGs in all detected samples. (B) Venn diagram for DEGs in response to cold and drought stress in *Zea mays* ssp. *mexicana* L. (C) Profiles of the numbers of Up- and down-regulated unigenes.

3.3.3 GO enrichment of DEGs

GO enrichment analysis was performed to investigate over-represented biological

processes or molecular functions involved in responses to cold or drought stress. A total of 45 and 89 GO terms were identified based on FDR < 0.05 as over-representations under cold and drought, respectively (Table 3.4, Table 3.5, Supplemental file 7).

Apparently, terms related to the response to abiotic stimuli, especially those to temperature and light, were significantly enriched in cold-treated *Zea mays* ssp. *mexicana* L. seedlings. Genes for heat shock factor proteins (HSF) such as hsf1 (c59237_g1), hsp3 (c60986_g3), hsf4 (c46749_g1), hsf7 (c54684_g2), hsf26 (c46143_g1), hsp81 (c56395_g1), and hsp101 (c58173_g1) were down-regulated under cold stress. With respect to light responsive genes those for phosphatase 2C50 (c57868_g1) and chloroplastic-like stress response protein (c62415_g1) were up-regulated under cold stress. Additionally, terms related to the regulation of gene expression, mostly at the level of transcription, were over-represented. Several genes for encoding transcription factors, AP2/EREBP (c48755_g1 and c45827_g1), NAC (c60421_g1 and c47289_g2), bZIP (c61581_g3 and c59299_g1), WRKY (c59902_g1, c38115_g1 and c63357_g2 as examples) and ethylene-responsive transcription factor (c56755_g1 and c63899_g2), were up-regulated under cold stress. Together, these observations emphasized the roles of transcriptional regulation of cold-related responses in this species.

Table 3.4 Enriched GO terms corresponding to biological processes and molecular functions under cold stress.

GO term ID	Term type	Term description	FDR
GO:0009408	P	response to heat	7.40E-10
GO:0009628	P	response to abiotic stimulus	9.20E-08
GO:0009644	P	response to high light intensity	9.20E-08
GO:0009266	P	response to temperature stimulus	3.80E-07
GO:0006355	P	regulation of transcription, DNA-dependent	3.80E-07
GO:0045449	P	regulation of transcription	3.80E-07
GO:0051252	P	regulation of RNA metabolic process	3.90E-07
GO:0031326	P	regulation of cellular biosynthetic process	9.40E-07
GO:0019219	P	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	9.40E-07
GO:0009889	P	regulation of biosynthetic process	9.40E-07
GO:0010556	P	regulation of macromolecule biosynthetic process	9.40E-07
GO:0051171	P	regulation of nitrogen compound metabolic process	1.20E-06
GO:0009642	P	response to light intensity	2.30E-06
GO:0030528	F	transcription regulator activity	2.90E-06
GO:0080090	P	regulation of primary metabolic process	3.30E-06
GO:0010468	P	regulation of gene expression	5.60E-06
GO:0031323	P	regulation of cellular metabolic process	5.60E-06
GO:0006351	P	transcription, DNA-dependent	6.10E-06
GO:0006350	P	transcription	6.10E-06
GO:0003700	F	transcription factor activity	6.50E-06
GO:0032774	P	RNA biosynthetic process	7.40E-06
GO:0060255	P	regulation of macromolecule metabolic process	1.60E-05
GO:0042542	P	response to hydrogen peroxide	5.90E-05
GO:0019222	P	regulation of metabolic process	6.70E-05
GO:0006950	P	response to stress	7.50E-05
GO:0033926	F	glycopeptide alpha-N-acetylgalactosaminidase activity	0.00038
GO:0003677	F	DNA binding	0.00046
GO:0000302	P	response to reactive oxygen species	0.00052
GO:0050794	P	regulation of cellular process	0.0013

GO:0042221	P	response to chemical stimulus	0.0019
GO:0016706	F	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	0.0035
GO:0050789	P	regulation of biological process	0.0038
GO:0050896	P	response to stimulus	0.0039
GO:0006560	P	proline metabolic process	0.005
GO:0065007	P	biological regulation	0.005
GO:0009416	P	response to light stimulus	0.0051
GO:0055114	P	oxidation reduction	0.0053
GO:0009314	P	response to radiation	0.0085
GO:0006970	P	response to osmotic stress	0.013
GO:0009415	P	response to water	0.019
GO:0005509	F	calcium ion binding	0.023
GO:0016070	P	RNA metabolic process	0.024
GO:0009753	P	response to jasmonic acid stimulus	0.027
GO:0009414	P	response to water deprivation	0.032
GO:0042401	P	cellular biogenic amine biosynthetic process	0.045

Note: P-biological process; F-molecular function

For the drought associated responses, however, there were a few differences. Under drought stress, DEGs were enriched for a total of 89 processes. Responses to stimuli, especially those to water deprivation, and regulation at the level of transcription were the most dramatic. Genes for the response to water deprivation were up-regulated under drought. These genes include dehydration-responsive element-binding protein 1D (c49699_g2 and c55715_g7), galactinol synthase gene (c60364_g1) and aquaporin PIP1-5 (c56750_g3 and c35748_g1). Genes involved in abscisic acid responses, such as EID1-like F-box protein (c57232_g1, c57232_g2 and c57232_g3), NAC domain transcription factor (c52045_g1), protein phosphatase 2C (c57868_g1, c50507_g1 and c60116_g4), and

stachyose synthase (c55229_g2), were up-regulated under drought.

To further identify the differences between responses induced by cold and drought in *Zea mays* ssp. *mexicana* L., a direct comparison was carried out between cold and drought-treated samples. A total of 2,514 assembled unigenes were identified by $|\log_2 change| >= 2$ and adjusted p-value < 0.005 (Figure 3.2C). GO enrichment analysis for these DEGs indicated that the differences between cold and drought induced responses were in relation to abiotic stresses of temperature and water, transcriptional regulation and protein phosphorylation processes (Supplemental file 5). The FDR value in relation to the abscisic acid related signaling pathway was 0.011 which was very close to the criteria of 0.01 (Table 3.5), suggesting that this pathway was also a major difference between the two datasets. Together, the enrichment patterns identified by directly comparing responses to cold and drought were in agreement with the above results obtained from comparing each in turn with the untreated control.

3.3.4 qRT-PCR verification

In order to validate the RNA-seq results, twelve DEGs with different expression patterns were selected for qRT-PCR analysis using gene-specific primers (Supplemental file 6). Among these DEGs, nine expressed under both cold and drought stresses, one regulated by cold only and two regulated by drought only were included. Eight of ten cold-responsive DEGs and night of eleven drought-responsive DEGs were confirmed by qPCR (Figure 3.3).

Table 3.5 Enriched GO terms corresponding to biological processes and molecular functions under drought stress.

GO term ID	Term type	Term description	FDR
GO:0009415	P	response to water	4.4E-12
GO:0004722	F	protein serine/threonine phosphatase activity	3.9E-11
GO:0009408	P	response to heat	4.1E-11
GO:0009414	P	response to water deprivation	8.6E-11
GO:0006470	P	protein amino acid dephosphorylation	2E-10
GO:0009628	P	response to abiotic stimulus	7.3E-10
GO:0009644	P	response to high light intensity	7.1E-09
GO:0004721	F	phosphoprotein phosphatase activity	9.3E-09
GO:0003700	F	transcription factor activity	9.3E-09
GO:0009266	P	response to temperature stimulus	2.6E-08
GO:0042221	P	response to chemical stimulus	2.9E-08
GO:0042578	F	phosphoric ester hydrolase activity	5.6E-08
GO:0030528	F	transcription regulator activity	5.6E-08
GO:0016791	F	phosphatase activity	6E-08
GO:0009738	P	abscisic acid mediated signaling pathway	6.3E-08
GO:0071215	P	cellular response to abscisic acid stimulus	4.6E-07
GO:0006950	P	response to stress	1.7E-06
GO:0009642	P	response to light intensity	2.7E-06
GO:0016311	P	dephosphorylation	2.7E-06
GO:0009737	P	response to abscisic acid stimulus	6.5E-06
GO:0042542	P	response to hydrogen peroxide	0.000021
GO:0050896	P	response to stimulus	0.000034
GO:0006350	P	transcription	0.000037
GO:0006351	P	transcription, DNA-dependent	0.000037
GO:0032774	P	RNA biosynthetic process	0.00004
GO:0016161	F	beta-amylase activity	0.000052
GO:0006355	P	regulation of transcription, DNA-dependent	0.000064
GO:0045449	P	regulation of transcription	0.000064
GO:0051252	P	regulation of RNA metabolic process	0.000067
GO:0031323	P	regulation of cellular metabolic process	0.000084
GO:0010556	P	regulation of macromolecule biosynthetic process	0.000094
GO:0050794	P	regulation of cellular process	0.000094
GO:0019219	P	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.000099
GO:0016160	F	amylase activity	0.00011
GO:0051171	P	regulation of nitrogen compound metabolic process	0.00012

GO:0031326	P	regulation of cellular biosynthetic process	0.00014
GO:0009889	P	regulation of biosynthetic process	0.00014
GO:0009788	P	negative regulation of abscisic acid mediated signaling pathway	0.0002
GO:0000302	P	response to reactive oxygen species	0.00021
GO:0080090	P	regulation of primary metabolic process	0.00025
GO:0009968	P	negative regulation of signal transduction	0.00025
GO:0023057	P	negative regulation of signaling process	0.00025
GO:0010648	P	negative regulation of cell communication	0.00025
GO:0010468	P	regulation of gene expression	0.00025
GO:0009416	P	response to light stimulus	0.00026
GO:0048585	P	negative regulation of response to stimulus	0.00029
GO:0019222	P	regulation of metabolic process	0.00029
GO:0016052	P	carbohydrate catabolic process	0.00035
GO:0009314	P	response to radiation	0.00036
GO:0006979	P	response to oxidative stress	0.00037
GO:0060255	P	regulation of macromolecule metabolic process	0.00038
GO:0009311	P	oligosaccharide metabolic process	0.00042
GO:0050789	P	regulation of biological process	0.00045
GO:0043687	P	post-translational protein modification	0.001
GO:0010029	P	regulation of seed germination	0.0011
GO:0009787	P	regulation of abscisic acid mediated signaling pathway	0.0011
GO:0065007	P	biological regulation	0.0012
GO:0009719	P	response to endogenous stimulus	0.0012
GO:0006796	P	phosphate metabolic process	0.0012
GO:0006793	P	phosphorus metabolic process	0.0012
GO:0009725	P	response to hormone stimulus	0.0015
GO:0003677	F	DNA binding	0.0017
GO:0000272	P	polysaccharide catabolic process	0.002
GO:0032870	P	cellular response to hormone stimulus	0.0024
GO:0005509	F	calcium ion binding	0.0032
GO:0009755	P	hormone-mediated signaling pathway	0.0033
GO:0048522	P	positive regulation of cellular process	0.0038
GO:0006464	P	protein modification process	0.0039
GO:0016788	F	hydrolase activity, acting on ester bonds	0.0041
GO:0071495	P	cellular response to endogenous stimulus	0.0042
GO:0010033	P	response to organic substance	0.0076
GO:0050793	P	regulation of developmental process	0.0096
GO:0016070	P	RNA metabolic process	0.0098
GO:0043412	P	macromolecule modification	0.013
GO:0051239	P	regulation of multicellular organismal process	0.014

GO:0009845	P	seed germination	0.02
GO:0005975	P	carbohydrate metabolic process	0.021
GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds	0.021
GO:0016567	P	protein ubiquitination	0.022
GO:0000151	C	ubiquitin ligase complex	0.025
GO:0006970	P	response to osmotic stress	0.026
GO:0071310	P	cellular response to organic substance	0.027
GO:0032446	P	protein modification by small protein conjugation	0.028
GO:0048518	P	positive regulation of biological process	0.028
GO:0004842	F	ubiquitin-protein ligase activity	0.032
GO:0006629	P	lipid metabolic process	0.033
GO:0019787	F	small conjugating protein ligase activity	0.037
GO:0070887	P	cellular response to chemical stimulus	0.038
GO:0016798	F	hydrolase activity, acting on glycosyl bonds	0.043

Note: P-biological process; F-molecular function

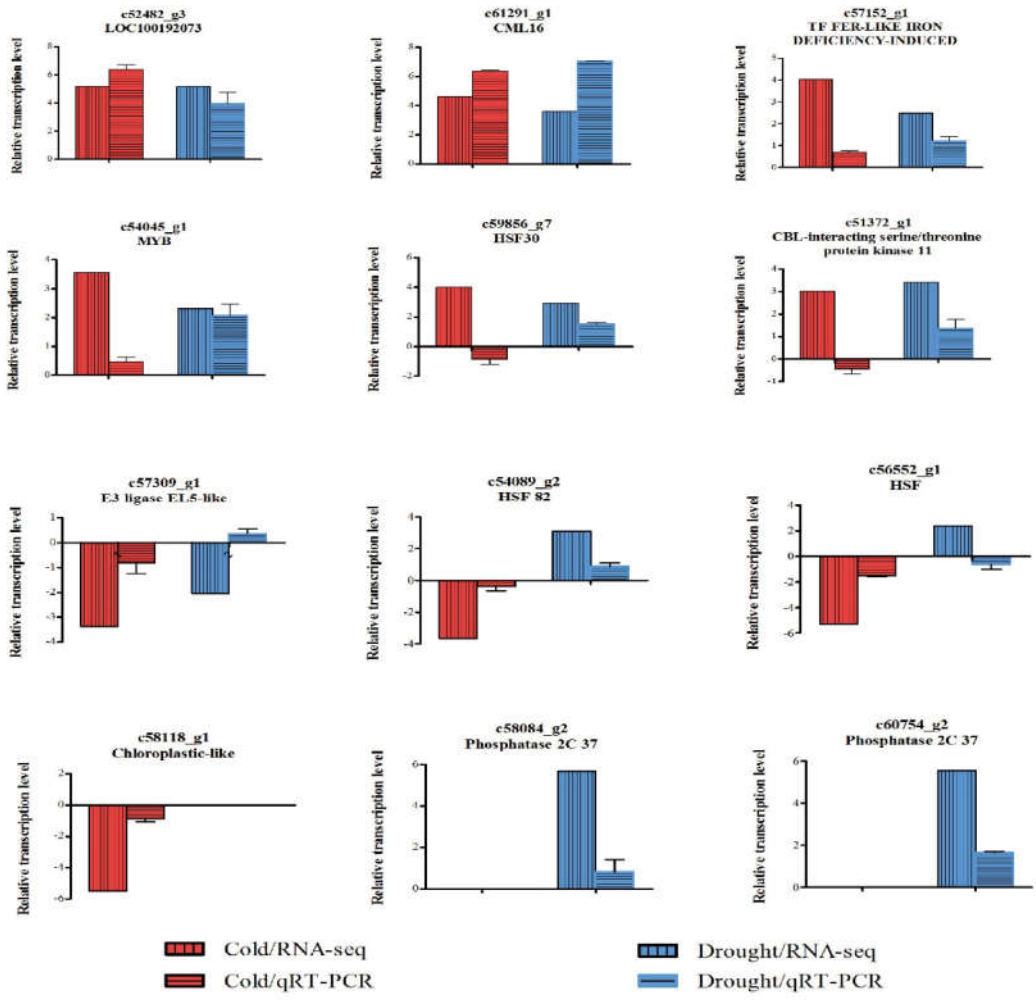

Figure 3.3 Verification for expressions of 12 selected DEGs.

*Relative expressions of the 12 genes in cold- or drought-treated *Zea mays* ssp. *mexicana* L. seedlings were tested by qRT-PCR. Transcription levels were normalized to that of actin and compared to the untreated control. Values of fold change are showed in mean ± SEM. Three independent replicates were performed for each gene. Fold changes of these genes detected in RNA-Seq are also showed for reference. Relative transcription levels were calculated by $\log 2^{-\Delta\Delta CT}$ method. The names of the closest putative orthologs are indicated for each gene, and the annotations are as following: c52482_g3, uncharacterized protein LOC_100192073; c61291_g1, calcium-binding protein CML16; c57152_g1, transcription factor FER-LIKE IRON DEFICIENCY-INDUCED; c54045_g1, MYB DNA-binding

domain superfamily protein isoform X1; c59856_g7, heat shock factor protein HSF30; c51372_g1, CBL-interacting serine/threonine-protein kinase 11 isoform X1; c57309_g1, E3 ubiquitin-protein ligase EL5-like; c54089_g2, heat shock protein 82; c56552_g1, 17.0 kDa class II heat shock protein; c58118_g1, photosystem I reaction center subunit N, chloroplastic-like; c58084_g2, protein phosphatase 2C37; c60754_g2, homeobox-leucine zipper protein ATHB-6.

3.4 Discussion

RNA-Seq technology has been successfully used at the level of transcriptomes or whole genomes analysis in many plants and crops (Garcia-Seco *et al.* 2015; Lu *et al.* 2010; Xia *et al.* 2011; Xu *et al.* 2012; Zhao *et al.* 2012). *Zea mays* ssp. *mexicana* L. is a subspecies of *Zea mays*, and it has a genome size of 2.58 Gb (2n=20) which is more than 20 times greater than the *Arabidopsis thaliana* (0.115 Gb) genome, 6 times greater than the rice (0.43 Gb) genome and equivalent to the *Zea mays* ssp. *mays* genome (2.73 Gb) (Hufford *et al.* 2012). The variety 8493, which serves as a representative of *Zea mays* ssp. *mexicana* L., is reported to grow well under 25 °C and can adapt to semi-arid and semi-humid environments (Huang *et al.* 1992). In this study, RNA-seq analyses were performed for *de novo* assembly of the transcriptome of *Zea mays* ssp. *mexicana* L. and identification of the differentially expressed genes (DEGs) in response to cold or drought stress.

3.4.1 An overview of the *Zea mays* ssp. *mexicana* L. transcriptome

By pooling the clean reads from all samples, we obtained a limited transcriptome which provided a profile the transcript dynamics under cold and drought stresses. The transcriptome identified 184,280 unigenes, and 251,145 transcripts which Blast analysis divided in protein coding and other genes. When growing under cold (4 °C) conditions, the change of gene expression was dramatic at the global level of transcriptome (Figure 3.2A, C), indicating that cold induces an extensive activation of transcription. Moreover, GO analysis (Table 3.4) suggested there was an activation of gene networks involving in cold/heat-induced responses. The drought stress used here, however, induced fewer

transcriptional changes, (only 15% as many) than cold (Figure 3.2C). These observations conformed the geographic distribution and the ideal growth conditions of *Zea mays* ssp. *mexicana* L. (Huang *et al.* 1992; Hufford *et al.* 2012). Similar observations have also been found in the elite maize inbred line Zheng 58 of *Zea mays* in which it was reported that the number of differentially expressed genes under drought stress was approximate 42% of that under cold (Shan *et al.* 2013), suggesting that the more sensitive response to cold rather than drought would be a conserved mechanism in *Zea*.

It is well known that gene expression in response to cold stress is somewhat different from that to drought stress (Shinozaki, Yamaguchi-Shinozaki & Seki 2003). In this study, only 194 DEGs are shared in stressed *Zea mays* ssp. *mexicana* L., (6.4% and 31.1% of cold- and drought-related DEGs, respectively). Among these, nearly 90% are regulated in a similar manner by both stresses, while only 15 DEGs are oppositely regulated (Supplemental file 4), indicating that there is a shared network to regulate the cold and drought induced responses in *Zea mays* ssp. *mexicana* L. On the other hand, specific regulations in response to cold or drought stress are also clearly visible in *Zea mays* ssp. *mexicana* L. The DEGs in response to cold are mostly enriched in processes of "Response to abiotic stimulus (GO:0009628)", especially the "Response to heat (GO:0009408)", and "Response to high light intensity (GO:0009644)" (Table 3.4), while the processes of "Response to water (GO:0009415)", "Response to water deprivation (GO:0009414)" and "protein amino acid dephosphorylation (GO:0004722)" are dominant in the collection of drought-induced DEGs (Table 3.5).

3.4.2 Transcriptional regulatory networks and signaling pathways involved in cold and drought induced responses.

Transcription factors play crucial roles in the regulation of target gene expression by specifically binding to cis-acting elements in the promoter regions (Agarwal *et al.* 2006b; Van Buskirk & Thomashow 2006). In this study, WRKY, CBL, MYB, NAC, bHLH, bZIP

and AP2/EREBP families that are known to be involved in stress tolerance in plants were identified. Members of WRKY (c56800_g2 and c56800_g1), CBL (c51372_g1 and c49176_g1), MYB (c54045_g1), bHLH (c48928_g1), bZIP (c59299_g1), AP2/EREBP (c51992_g7, c48755_g1) family are up-regulated by both stresses of cold and drought (Table 3.2), suggesting shared upstream pathways for signal transduction and regulation under these stimuli. From those down-regulated TF proteins, only one NAC (c57823_g1) protein is consistently down-regulated by both stresses, which is similar to the findings in *Arabidopsis* and rice (Lu *et al.* 2007; Takasaki *et al.* 2010) and suggests a negative regulation mechanism in *Zea mays* ssp. *mexicana* L. It is notable that some previously uncharacterized TFs are significantly up- or down-regulated in response to cold or drought only. For example, a member of TUB family (c61146_g4), was found in cold-induced DEGs set but not in the drought-induced set. This is similar to what has been observed in *V. amurensis* (Xu *et al.* 2014c). In addition, some members in the ULT (c53036_g1), BIM2 (c64573_g2) and DBP (c51832_g3) families are found to be up-regulated by cold only (Table 3.2). The AP2/ERF transcription factor family comprises four major subfamilies, namely AP2, ERF, RAV and dehydration-responsive element binding proteins (DREB/CBF) (Shinozaki, Yamaguchi-Shinozaki & Seki 2003). The roles of DREBs/CBFs in stresses induced response have been well established in numerous plants including *A. thaliana* (Ma *et al.* 2014) , rice (Challam *et al.* 2015), maize (Liu *et al.* 2013) and soybean (Kidokoro *et al.* 2015). The DREB1s/CBFs can activate the downstream cold-responsive genes via specific binding to the DRE/CRT cis-acting element in their promoters, and expressions of these genes are positively correlated with cold tolerance (Mizoi, Shinozaki & Yamaguchi-Shinozaki 2012). In *Zea mays* ssp. *mexicana* L., three DREB genes (c51992_g7, c49699_g1, c49699_g2) were found to be significantly induced by both cold and drought stresses, suggesting that these proteins might functionally act in *Zea mays* ssp. *mexicana* L. during cold and drought stresses.

Signaling components such as kinases participate in numerous processes, including

cell division, developmental programs, hormonal responses, and drought, salinity and reactive oxygen species (ROS) signaling responses (Wang *et al.* 2015a). In particular, the MAPK cascade, serving as a signaling joint for various abiotic stress, positively regulates cold acclimation in plants (Chinnusamy, Zhu & Sunkar 2010). In *Arabidopsis*, the AtMEKK1/ANP1 (MAPKKK)-AtMKK2 (MAPKK)-AtMPK4/6 (MAPK) mitogen-activated protein kinase cascade is necessary for cold acclimation, and AtMKK2 (MAPKK) was identified as a key signal transducer. In addition, overexpression of AtMKK2 induces the cold responsive genes CBF2 and CBF3 and improves cold tolerance in *Arabidopsis* (Teige *et al.* 2004b). In this study, one unigene coding for a MAP kinase (c14147_g1) and another one for a MAPKK protein (c62016_g1) were up-regulated by cold stress only, suggesting that the MAPK cascade may be involved in response to cold in *Zea mays* ssp. *mexicana* L.

3.4.3 *ICE1-CBF* pathway genes involved in cold and drought stresses

Cold induced responses come down to different metabolic pathways, gene regulations and cell compartments. The ICE1-CBF-COR transcriptional cascade is a highly conserved module which is activated in response to cold stress (Lissarre *et al.* 2010; Zhu *et al.* 2007), and has been demonstrated to have essential roles in response to cold, drought, and other abiotic stresses (Peng *et al.* 2015; Zhou *et al.* 2011). ICE1 is a MYC-type transcription factor that binds to the MYC recognition cis-elements (CANNTG) in the promoter of CBF3/DREB1A protein to activate the expression of CBF3/DREB1A, which consequently regulates 40% of COR genes and 46% of cold-regulated transcription factor genes (Lee, Henderson & Zhu 2005). In *Arabidopsis*, three CBF proteins, namely CBF1, 2 and 3, are induced by low temperature but not by abscisic acid (ABA) or dehydration (Medina *et al.* 1999a). *CBF1* and *CBF3* can respond to cold at the same time, but the response of the *CBF2* lags behind. *CBF2* negatively regulates the expression of *CBF1* and *CBF3* during cold acclimation, while *CBF1* and *CBF3* are not involved in regulation of other *CBF* genes. CBF1/CBF3 have lower cold acclimation ability and play a subsidiary function in the

CBF/DREB1 pathway and cold acclimation (Gilmour, Fowler & Thomashow 2004; Kim *et al.* 2015; Novillo, Medina & Salinas 2007). Differences in the functions of CBFs in mangrove *Avicennia marina,* European bilberry (*Vaccinium myrtillus*) and *Brassica rapa* have been observed. For example, *CBF4* is regulated by drought and ABA but not by cold (Haake *et al.* 2002; Lee *et al.* 2012b; Oakenfull, Baxter & Knight 2013; Peng *et al.* 2013). In this study, two putative ICE1 homologs (c60792_g1, c13919_g1) were identified in this subspecies. However, expressions of these genes were not affected by cold. A possible explanation is that the ICE1 homolog may be induced rapidly and transiently, and is attenuated in a short time, making it undetectable. In addition, CBF6 has been found to play a role in freezing tolerance in *Festuca pratensis* (Ergon *et al.* 2016). In our study, a CBF6 (c57018_g4) homologous gene was found to be up-regulated in response to cold and drought stresses, suggesting that this CBF6 homologous gene might play roles in stress resistance in *Zea mays* ssp. *mexicana* L.

3.4.4 Trehalose may be a common regulator of the response to cold and drought stresses.

Trehalose is widely found in various organisms and is a non-reducing disaccharide formed by linking two glucose units with an a,a-1,1-glycosidic linkage (Wang *et al.* 2005). The synthetization of trehalose involves two-steps. The first step is the formation of trehalose-6-phosphate (T6P) from UDP-glucose and glucose-6-phosphate, which is catalyzed by trehalose-6-phosphate synthase (TPS). The second step is the generation of trehalose through dephosphorylation which is catalyzed by trehalose-6-phosphate phosphatase (TPP) (Zang *et al.* 2011). The genes coding for the TPS and TPP enzymes are thus important for the regulation of trehalose production (Ilhan, Ozdemir & Bor 2015). Transgenic plants harboring TPS or TPP genes have enhanced tolerance to abiotic stresses by stabilizing dehydrated enzymes, as well as by protecting protein and cellular membranes structures (Nuccio *et al.* 2015). In our study, a key trehalose-6-phosphate phosphatase (TPP) gene (c45624_g2) was also found to be up-regulated under both cold and drought stresses in *Zea*

mays ssp. *mexicana* L. This is in agreement with the positive roles of trehalose in tolerating low temperature and drought stresses.

3.4.5 Phytohormone dependent pathways.

Plant hormones play vital roles in adaption to stressful environments by regulating growth, development, nutrient allocation and source/sink transitions. Previous studies showed that different types of plant hormones, including abscisic acid (ABA), cytokinins (CKs), jasmonic acid (JA), gibberellins (GAs), brassinosteroids (BRs), ethylene (ETH) and auxin, have functions in regulating freezing tolerance by CBF-dependent or -independent pathways (Shi, Ding & Yang 2015). ABA is the key phytohormone used in plants for regulation of the responses to abiotic stresses. As an example, the secondary hyperosmotic stress derived from drought or salt stress causes the accumulation of ABA which in turn rapidly elicits downstream responses in plants (Zhu 2016). ABA accumulation can cause stomatal closure, thereby reducing water loss and eventually restrict cellular growth. At the molecular level, ABA regulates gene transcription, protein synthesis, signaling pathways, ion transport (and the transport of other organic molecules) and the production of important protectants against dehydration and photoinhibition (Boursiac *et al.* 2013; Raghavendra *et al.* 2010). Cold stress is also reported to induce ABA biosynthesis, and the exogenous application of ABA improves the cold tolerance of plants (de Zelicourt, Colcombet & Hirt 2016). ABA biosynthesis is one of the important steps used by plants in responding to stresses via the ABA-dependent pathway. The 9-cis epoxycarotenoid dioxygenase 1 (NCED1) is regarded as a key rate-limiting enzyme in ABA biosynthesis. It was originally identified from maize *viviparous 14* mutants, and is responsible for cleavage of the ABA precursor C40-cis-epoxycarotenoids, to either 9-cis-neoxanthin, 9-cis-violaxanthin or both to produce xanthoxin, the direct C15 precursor of ABA (Riahi *et al.* 2013). In *Zea mays* ssp. *mexicana* L., a homolog of NCED1 (c63716_g5) gene was identified. It was up-regulated by drought (Supplemental file 4), which is consistent with results reported previously in *Arabidopsis* (Behnam *et al.* 2013) and *Phaseolus vulgaris* L. (Qin & Jan

1999), suggesting conserved regulation of this drought induced response in *Zea mays* ssp. *mexicana* L. It is notable that this NCED1 gene was also activated by cold stress (Supplemental file 4), which is similar to recent observations for OsNCED1 and OsNCED3 (Maruyama *et al.* 2014), suggesting that the ABA-dependent pathway plays roles in response to cold and drought in *Zea mays* ssp. *mexicana* L.

PP2C proteins are also reported to be involved in ABA-dependent regulations of stress induced responses, such as the seventy-six phosphatase 2C (PP2C-type) proteins which have been identified from *Arabidopsis*. Most of these (PP2C-type) proteins fall into ten groups (A–J) (Schweighofer, Hirt & Meskiene 2004). ABA signaling can be mediated by type 2C protein phosphatases (PP2Cs) such as HAB1 and ABI2, which inhibit stress-activated SnRK2 kinases (Nakashima *et al.* 2009). When directly comparing the expression levels under cold with those under drought conditions in *Zea mays* ssp. *mexicana* L., four candidate PP2C genes, including c52482_g3, c52482_g6, c62830_g2, and c57868_g1, showed up-regulated patterns. A putative protein kinase SnRK1 (c57228_g1), however, was down-regulated only under cold stress. These results taken together suggest that PP2C protein is involved in ABA-dependent signaling and affects the regulation of cold induced responses in *Zea mays* ssp. *mexicana* L.

Previous studies on *Zea mays* ssp. *maize* L. transcript profile under cold, salt and drought stresses indicate that the abundance of bioactive GAs was reduced and that the negative regulatory factor DELLAs were accumulated (Shan *et al.* 2013), suggesting a negative effects of gibberellin in stress induced responses. This is in disagreement with what we observed in cold-stressed *Zea mays* ssp. *mexicana* L. Genes for the GAs receptor GID1L2 precursor (c47232_g1), the GA 2-beta-dioxygenase (c57017_g1, c33506_g1, c57017_g2) and the GA20 oxidase (c63822_g4, c49254_g1, c56760_g2) that are involved in GAs synthesis or signaling were up-regulated by cold, suggesting a positive role of gibberellin in this subspecies in response to cold stress. Thus, GAs could differentially regulate the responses to cold in these two close *Zea mays* subspecies. Future comparative

study will provide better understanding of the molecular basis of the GA associated response to cold in *Zea mays*.

3.5 Conclusion

In summary, this study provided an overview of the transcriptome of *Zea mays* ssp. *mexicana* L., and the changes of transcript abundance in response to cold or drought stress. Less than a half of the reported *Zea mays* proteome have homologs in *Zea mays* ssp. *mexicana* L., indicating that specific regulation networks or mechanisms exist in these close relatives. DEGs responsive to cold or drought conditions serve as candidates for further study on tolerance related gene networks in *Zea mays* ssp. *mexicana* L. The effects of drought and cold reported here have arisen from a limited range of potential types and severities of stress. Thus it should be noted that a greater range of treatments for (e.g. timing, severity, frequency, source) need to be examined in future studies to provide more clues for understanding the adaptation and tolerance mechanisms in this species.

Chapter 4 A novel *Zea mays* ssp. *mexicana* L. MYC-type ICE-like transcription factor gene ZmmICE1, enhances freezing tolerance in transgenic *Arabidopsis thaliana*

Abstract

The annual *Zea mays* ssp. *mexicana* L., a member of the teosinte group, is a close wild relative of maize and thus can be effectively used in maize improvement. In this study, an *ICE*-like gene, *ZmmICE1*, was isolated from a cDNA library of RNA-Seq from cold-treated seedling tissues of *Zea mays* ssp. *mexicana* L. The deduced protein of *ZmmICE1* contains a highly conserved basic helix-loop-helix (bHLH) domain and C-terminal region of *ICE*-like proteins. The ZmmICE1 protein localizes to the nucleus and shows sumoylation when expressed in an *Escherichia coli* reconstitution system. In addition, yeast one hybrid assays indicated that ZmmICE1 has transactivation activities. Moreover, ectopic expression of *ZmmICE1* in the *Arabidopsis ice1-2* mutant increased freezing tolerance. The *ZmmICE1* overexpressed plants showed lower electrolyte leakage (EL), reduced contents of malondialdehyde (MDA). The expression of downstream cold related genes of *Arabidopsis* C-repeat-binding factors (*AtCBF1*, *AtCBF2* and *AtCBF3)*, cold-responsive genes (*AtCOR15A* and *AtCOR47)*, kinesin-1 member gene (*AtKIN1)* and responsive to desiccation gene (*AtRD29A)* was significantly induced when compared with wild type under low temperature treatment. Taken together, these results indicated that *ZmmICE1* is the homolog of *Arabidopsis* inducer of CBF expression genes (*AtICE1/2)* and plays an important role in the regulation of freezing stress response.

Keywords *ZmmICE1*; Transcription factor; Transgenic *Arabidopsis thaliana*; Freezing tolerance; *Zea mays* ssp. *mexicana* L.

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4.1 Introduction

Zea mays ssp. *mexicana* L., a member of teosintes, is a wild annual grass native to high altitude in northern and central Mexico (altitude 1,600~2,700 m) with a large spikelet trait and is a close wild relative of cultivated maize (*Zea mays* ssp. *mays* L.). Crosses between these two subspecies have normal chromosome pairing in meiosis and are generally fertile (Fukunaga *et al.* 2005). It is an important genetic material both for the improvement of agronomic characteristics of maize and studies on teosinte genetics, genome evolution and breeding (Takahashi *et al.* 1999; Wang *et al.* 2008). *Zea mays* ssp. *mexicana* L. has a stronger growth and regeneration ability, more tillers, higher protein content in the kernel and more dominant resistance to many fungal diseases than cultivated maize and can be used to feed livestock directly or be reserved as silage or hay (Fang *et al.* 2012; Fukunaga *et al*. 2005). However, most lines of *Zea mays* ssp. *mexicana* L., originated from subtropical areas and are sensitive to low temperature stress (Hincha & Zuther 2014). Thus, an understanding of the molecular mechanisms of key cold tolerance genes of *Zea mays* ssp. *mexicana* L. is one of the key objectives for improving its yield and expanding its geographic districts of production.

Low temperature is one of the major environmental factors that impact the geographical distribution plant species. It can also lead to a decrease of crop quality and productivity (Puyaubert & Baudouin 2014; Viswanathan & Zhu 2002). A series of molecular response mechanisms of plant signal transduction pathways are activated with low temperature stress. Many complex regulator factors participate in the cold signaling process. These factors include receptors, secondary signals (ROS, ABA), second messengers (Ca^{2+}, IP3, cAMP), the kinase cascade pathway (*MAPK* and *CDPK*), transcription factors (*CBF/DREB*, *bZIP*, *MYB/MYC*) and stress response genes (*ROS*) (Winfield *et al.* 2010). Other genes, such as *ICE, HOS1, MYCR, DREB, CBF/DREB1, DREB2, AREB,* are also involved in the plant cold response regulating network. However, an accurate description of the regulation mechanisms for the activation of *ICE*-like genes

and for transducing signals from environmental detection and the second messengers to *ICE*-like genes are still not clear (Dong *et al.* 2006; Miura *et al.* 2007).

The *ICE1-CBF-COR* transcriptional cascade has been reported to be the primary cold acclimation signal regulatory pathway (Chinnusamy, Zhu & Zhu 2007; Lissarre *et al.* 2010; Zhu *et al.* 2007). Mutant *Arabidopsis* with the transcription factor Ice1 is sensitive to both chilling and freezing stresses. Freezing tolerance can be increased by overexpression of this gene (Chinnusamy *et al.* 2003). *ICE1* controls *CBF3/DREB1A* and many other *COR* genes by binding to the *MYC* recognition cis-elements (CANNTG) in the promoter of *CBF3/DREB1A* thus inducing the expression of *CBF3/DREB1A* and its regulons during cold acclimation. Nearly 40% of *COR* genes and 46% of cold-regulated transcription factor genes are regulated by *ICE1* (Lee, Henderson & Zhu 2005). Additionally, the *Arabidopsis* gene *AtICE2*, is a positive regulator belonging to the basic helix-loop-helix (bHLH) family, has been identified to activate *AtCBF1* (Xu *et al.* 2014c). Many different types of *ICE*-like genes with similarly conserved domain structures have been detected and cloned in different higher plants and the functions of *ICE1* have been confirmed by transgenic experiments (Dong *et al.* 2013; Xiang *et al.* 2013; Xu *et al.* 2014a). Heterologous overexpression of *Arabidopsis thaliana ICE1* has increased chilling tolerance of cucumber (Liu *et al.* 2010). Transgenic tobacco over-expressing *VaICE1* from *Vitis amurensis*, with its basic helix-loop-helix domain, has also shown a better chilling tolerance and survival ability under cold stress through improved activities of superoxide dismutase, peroxidase and catalase, as well as increased chlorophyll yield (Dong *et al.* 2013).

In our current study, we investigated an *ICE*-like gene *ZmmICE1* from *Zea mays* ssp. *mexicana* L. which has a similar structure to the *ICE* gene family. The function of *ZmmICE1* was profiled in transformed *Arabidopsis* plants by transient and stable expression assays.

4.2 Materials and methods

4.2.1 Plant materials, growth condition and RNA isolation

The seeds of *Zea mays* ssp. *mexicana* L. variety "8493" were obtained from the Guangdong Provincial Key Laboratory of Biotechnology for plant development research at the School of Life Science of the South China Normal University (Guangzhou, China). Plump seeds were sterilized and planted in plastic boxes filled with soil substrates (Jiffy, Netherlands), following the germination methods described in the CIMMYT operation manual (Taba *et al.* 2004b). The boxes were placed in a climate control cabinet (RXZ 500-C, JIANGNAN Instrument) with the temperature set at 25 °C; a 10-h light/14-h dark photoperiod, and a humidity of 60%. Twenty-one day old seedlings were treated with 4 °C cold stress for 12 h before being frozen in liquid nitrogen for total RNA isolation. Total RNA of mixed samples was extracted by TRIzol® reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. RNA was purified with RNase-free DNase I and used for reverse transcription by PrimeScript™ RT reagent Kit (Takara, Japan). *Arabidopsis thaliana* mutant-type (*ice1-2,* Salk_003155) was grown for genetic transformation and WT-type (*Columbia 0*) as used as a control (22 °C under a 16-h light/8-h dark photoperiod, humidity of 40%).

4.2.2 Isolation of the full-length cDNA of *ZmmICE1* and bioinformatics analysis

To determine the expression profiles of *ZmmICE1* of *Zea mays* ssp. *mexicana* L. and *Arabidopsis* plants, total RNAs and cDNA were prepared as described above. Based on our unigenes *de novo* sequence by RNA-Seq and nucleotide sequence BLAST results, the full CDS sequence was confirmed. PCR was performed using primer pairs specific for the full-length *ZmmICE1* gene, with forward primer 5'-AAGCTTATGGACAACTCGAAGGAGGTAAAGC-3'; and reverse primer 5'-TCTAGATTACATTGCATTGTGGAGACCGG-3' (the added restriction sites for *Hind III* and *Xba I* are underlined, respectively). The PCR program was as follows: 98 °C, 3 min; 30 cycles, 98 °C, 30 s; 60 °C, 30 s; 68 °C, 60 s; 68 °C, 10 min. The completed *ZmmICE1*

was cloned into the *pEASY*®-Blunt Simple Cloning Vector (TransGen Biotech, GuangZhou) for sequencing. The PCR products were then cloned into Pba002-myc vector.

The online computer program pI/MW (http://web.expasy.org/compute_pi/) was used to predict the theoretical pI (isoelectric point) and MW (molecular weight) of the ZmmICE1 protein. A phylogenetic tree was constructed based on the deduced amino acid sequences of *ICE*-like genes from a range of plant species by MEGA 5.1 with 500 bootstrap tests by Neighbor-joining tree. ClustalX 1.8 and DNAman 8.0 alignment were employed for deducing the ZmmICE1 amino acid sequence homology alignment. Predicted molecular model building of ZmmICE1 was carried out using the phyre2 online server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

To determine the expression profiles of *ZmmICE1* in *Zea mays* ssp. *mexicana* L., total RNA were extracted as describe above. The operation methods are the same as that for the analysis of cold responsive genes that regulate *ZmmICE1*. Semi-quantitative RT-PCR was performed to assess the expression of *ZmmICE1* over a cold stress time course at 4 °C. The *ZmmICE1* qRT-PCR primers are 5′-AGGGCACGCAGTCAGTATCC-3′ and 5′-CGCGCACTCAGCTCGGAA-3′. *Zea mays* 18S rRNA was used as a loading control using the following primers: 5’-CCTGCGGCTTAATTGACTC-3’ and 5’-GTTAGCAGGCTGAGGTCTCG-3’. Three replicates were performed for each semi-quantitative RT-PCR reaction.

4.2.3 Subcellular localization of ZmmICE1 protein

To construct yellow fluorescent protein (YFP) translational fusion vectors, full-length *ZmmICE1* was PCR amplified with the primers 5’-GAATTCATGGACAACTCGAAGGAGGTAAAGC-3’ (*EcoR I*) and 5’-ACTAGTTTACATTGCATTGTGGAGACCGG-3’ (*Spe I*), and fused in-frame downstream of the YFP reporter gene in modified pBluescript II Phagemid vector. The empty vector containing only YFP sequence was used as a positive control. The concentration of isolated plasmids was ~1 μg/μl. YFP::ZmmICE1 fusion proteins were transiently expressed in protoplasts of

Arabidopsis, which are derived from rosette leaves according to the protocol from Jen Sheen's laboratory (Yoo, Cho & Sheen 2007). The transformed protoplasts were grown for 48 h at 23 °C and the subcellular localization of YFP fusion protein was observed under a Zeiss LSM 710 laser (Germany) scanning microscope with 514 nm for excitation and 530–600 nm for emission. The chlorophyll auto-fluorescence was also recorded. The photos from YFP, chloroplast, and bright field channels were merged.

4.2.4 Transcriptional activation assay of *ZmmICE1* in yeast

For transcriptional activation analysis, a full-length *ZmmICE1* was PCR amplified with the primers 5'-GAGGACCTGCATATGATGGACAACTCGAAGGAGGTAAAGC-3' (*Nde I*) and 5'-GATCCCCGGGAATTCTTACATTGCATTGTGGAGACCGG' (*EcoR I*), and cloned into the pGBKT7 vector (Clontech, Mountain View, CA, USA) to create the pGBKT7-*ZmmICE1* constructs. The positive control pGBKT7-53 + pGADT7-T (pGBKT7-53 encodes the Gal4 DNA-BD fused with murine p53, whereas pGADT7-T encodes the Gal4 AD fused with SV40 large T-antigen), and the negative control pGBKT7-lam + pGADT7-T, the empty contrast pGBKT7 + pGADT7 and the pGBKT7-*ZmmICE1* + pGADT7 were individually transformed into the AH109 yeast strain using the lithium acetate method according to the manufacturer's protocol. The transformed cells were plated onto both SD/-Leu/-Trp, SD/-Leu/-Trp/-His/0.5 mM 3-AT and SD/-Leu/-Trp/-His/X-a-gal media. After incubation at 28 °C for 3 days, the transcriptional activation ability of each transformant was detected by their survival and β-galactosidase activity.

4.2.5 Western blot protocol and *in vitro* sumoylation assay

In order to analyse the ZmmICE1 sumoylation process *in vitro* prokaryotic expression system, full-length *ZmmICE1* was PCR amplified with the primers 5'-GAATTCGATGGACAACTCGAAGGAGGTAAAGC-3' (*EcoR I*) and 5'-GTCGACTTGTCATCATCGTCCTTGTAGTCCATTGCATTGTGGAGACCGG-3' (*Sal I*). We cloned *ZmmICE1* into the prokaryotic expression vector pCDFDuet-1 (Novagen,

Germany) to generate a fusion vector with a FLAG tag which could be expressed in bacteria carrying *pET28-SAE1a-His-AtSAE2* (E1) and *pACYCDuet-1-SCE1-SUMO1 (GG)* (E2 and SUMO1). The transformed cells were cultured in LB medium with required antibiotics to an OD 600 of 0.5 and induced by 0.2 mM IPTG. After incubation for 12 h at 25 °C, cells were harvested and used for immunoblotting. The resultant proteins were separated with SDS-PAGE and blotted onto a PVDF membrane (Immobilon, USA). The gel blot was probed with anti-Flag (TransGen, China) antibodies and visualized by chemiluminescence using the ECL plus kit (Cwbio, China) according to the manufacturer's instructions.

4.2.6 Generation of *Arabidopsis* plants with complementary overexpression of *ZmmICE1*

The 35S::*ZmmICE1* recombinant plasmid was introduced into *Agrobacterium tumefaciens EHA105* by the infection method and transformed into *Arabidopsis thaliana* (Valvekens, Van Montagu & Van Lijsebettens 1988). The seeds of transformed plants were selected after being sown on MS culture plates supplemented with spectinomycin (2 μg/ml) and vernalization for 3 days. Resistant seedlings were transferred to soil. Transformed plants were identified with specific primer PCR, and their seeds were harvested separately. T3 homozygous seeds were identified by BASTA resistance and used for further experiments. For RT-PCR analyses, 2 μg sample of total RNA was used as a template for the first strand cDNA synthesis with M-MLV reverse transcriptase and an Oligo (dT) 18 primer (TaKaRa, Japan). *Atactin* specific primer 5'-GGCTGGATTTGCTGGAGATGATGC-3' and reverse primer 5'-CAATTTCTCGCTCTGCTGAGGTGG-3' were used as an internal control. RT-primer of *ZmmICE1* 5'-CGCAGACAGCGGCACAGCAG-3' and the reverse primer 5'-CGCGCACTCAGCTCGGAA-3' were used for RT-PCR.

4.2.7 Stress tolerance assay

For the freezing treatment analysis, sterilized seeds of wild type and T3 complementary

ice1-2 overexpression transgenic *Arabidopsis* mutants were germinated and grown on MS medium on a glass plate for 7 days after 72 h of vernalization, then transferred to plastic pots with a mixture of soil substrates: vermiculite (3:1, v/v) (Jiffy, Netherlands, http://www.jiffygroup.com/en/substrates/) for two weeks under 22 °C with a light/dark cycle of 16 h /8 h. For the freezing assay, the three-week-old seedlings of the transgenic complementary plants with a uniform growth status were transferred to a climate control box (GXZ-0450, JIANGNAN Instrument) at -6 °C for 8 h under continuous dim light conditions (2.5 μmol m^{-2} s^{-1}) and subsequently returned to normal conditions for recovery growth for 7 d. Control plants (wild type) were continuously grown under normal conditions. The freezing treatment experiment was repeated three times. The treated and untreated leaves were collected for future physiological assay.

4.2.8 Measurement of electrolyte leakage, malondialdehyde and proline content

More than 300 three-week-old *Arabidopsis* seedlings (control and transgenic) of each line were used for cold stress treatment under -2 °C for 48 h. Treated leaves were harvested to analyze three basic biochemical features related to cold tolerance: electrolyte leakage (EL), proline content (Pro) and malondialdehyde (MDA) content (Campos *et al.* 2003; Hadi, Gilpin & Fuller 2011; Jin *et al.* 2016).

For determination of EL, leaves (0.8×0.8 for each sample) were placed into 15 ml deionized water and shaken for 1 h at 100 rpm at room temperature before measuring the initial electronic conductance (E1). Subsequently, the mixture was heated to 100 °C for 30 min and then cooled to room temperature to re-determine the final electronic conductance (E2). The electronic conductance (E0) of deionized water was used as the control. The EL was calculated by the formula: EL = (E1-E0) / (E2-E0) × 100% (Armstrong *et al.* 2015).

MDA content was estimated using a thiobarbituric acid reactive substances (TBARS) assay. Briefly, the fresh homogenized leaves (0.5 g) were ground with quartz sand in 10 ml of trichloroacetic acid (TCA) and subsequently centrifuged at 4,000 rpm for 10 min. 2.0 ml of 0.67% (w/v) TBA (thiobarbituric acid) was added into 2 ml of the supernatant and

subsequently heated at 100 °C for 15 min, cooled and shaken quickly, and then centrifuged at 4,000 rpm for 5 min. The supernatant and 0.67% (w/v) TBA liquid (control) were measured for the absorbances at 532 nm (A_{532}), 600 nm (A_{600}), and 450 nm (A_{450}) using a spectrophotometer (UV-2450, SHIMADZU). MDA content was calculated using the formula: C (MDA content, umol/L) = $6.45(A_{532}-A_{600})-0.56\times A_{450}$ (Yan *et al.* 2014).

Proline content was determined using acid-ninhydrin reagent and glacial acetic acid. The fresh homogenized leaves (0.5 g) were ground with 3% sulphosalicylic acid and then extracted with 5 ml of 3% sulphosalicylic acid at 100 °C for 10 min with shaking. The proline extracting solution was filtered and 2 ml of solution was collected. The solution was mixed with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin reagent (2.5% (w/v) ninhydrin, 60% (v/v) glacial acetic acid, and 40% 6 M phosphoric acid) and incubated at 100 °C for 30 min. After cooling, 4 ml toluene was added and the solution was shaken for 30 s, then left standing for 2 h. The absorbance of the organic phase was determined at 520 nm. Proline content was then determined against the constructed standard curve (Li *et al.* 2004).

4.2.9 Analysis of cold responsive genes regulated by *ZmmICE1*

Total RNA was extracted from three-week-old control (WT and *ice1-2*) and T3 transgenic *Arabidopsis* leaves treated at 0 °C for 0, 12, 48 h by TRIzol® reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. cDNA was produced from 1 μg RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser, according to the manufacturer's instructions and quantitative real-time PCR was performed using the SYBR *Premix Ex Taq*™ kit (TakaRa) in the BIO-RAD CFX96 sequence detection system. The reaction was carried out in a 96-well plate with 3 replications. The 20 μl reaction system included SYBR *Premix Ex Taq* 10 μl, 0.4 μl of each primer (10 μM stock), 1 μl cDNA template and 8.2 μl ddH_2O. Two-step real time PCR reactions were used with the following steps: 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 30 s, and 95 °C for 5 s, melt curve 65 °C to 95 °C with increments

of 0.5 °C and then data acquisition. The downstream genes of the cold response pathway such as *AtCBF1*, *AtCBF2*, *AtCBF3*, *AtCOR15A*, *AtCOR47*, *AtKIN1* and *AtRD29A* were measured in *ZmmICE1* transgenic and control plants, respectively. The specific primers were: 5′-AGGGCACGCAGTCAGTATCC-3′ and 5′-CGCGCACTCAGCTCGGAA-3′ for *ZmmICE1*; 5′- AGGGCACGCAGTCAGTATCC-3′ and 5′-CGCGCACTCAGCTCGGAA-3′ for *AtCBF1*; 5′-GACCTTGGTGGAGGCTATTT-3′ and 5′-ATCCCTTCGGCCATGTTATC-3′ for *AtCBF2*; 5′-GACGTTGGTGGAGGCTATTT-3′ and 5′-AGCATCCCTTCTGCCATATTAG-3′ for *AtCBF3*; 5′-GCAGATGGTGAGAAAGCGAA-3′ and 5′-GGCATCCTTAGCCTCTCCTG-3′ for *AtCOR15A*; 5′-GGCTGAGGAGTACAAGAACAA-3′ and 5′-ACAATCCACGATCCGTAACC-3′ for *AtCOR47*; 5′-GCAATGTTCTGCTGGACAAG-3′ and 5′-TCCTTCACGAAGTTAACACCTC-3′ for *AtKIN1*; 5′-GCTTTCTGGAACAGAGGATGTA-3′ and 5′-CGACTCTTCCTCCAACGTTATC-3′ for *AtRD29A*. Primers for *β-tubulin* were 5′- AAGATTCGTCCCACGCG-3′ and 5′-TCCTTTAGCCCAATTGTTACC-3′ was used as the standard control to normalize all data. Each experiment was repeated three times.

4.3 Results

4.3.1 Isolation and characterization of *ZmmICE1* gene

We identified a putative *Zea mays* ssp. *mexicana* L. *ICE*-like gene based on the conserved domains of *ICE* family homology in several plant species. The full length cDNA sequence of the *ZmmICE1* gene was amplified. The total number of base pairs of complete cDNA of *ZmmICE1* is 1,122 bp and the cDNA encodes a predicted protein of 373-amino acids. The predicted molecular weight of ZmmICE1 is 39.35 kDa with a pI of 5.12. The complete ZmmICE1 protein showed 87% homology with maize putative HLH DNA-binding domain superfamily protein, which suggested that *ZmmICE1* is an ortholog of the *ICE* family.

After a BLAST search with different plants sequences, the highest query coverage sequence was extracted for sequences and homology analysis. The result indicated that

ZmmICE1 contains the same conserved bHLH domain and C-terminal region as other *ICE*-like proteins (Figure 4.1A). In addition, a potential SUMO binding domain has been reported previously in *Arabidopsis* ICE1 protein (Miura & Hasegawa 2008) and the conserved key residue serine (K393) were confirmed in the deduced amino acids, respectively. To investigate the evolutionary relationship among the ICE-like proteins, a phylogenetic tree was constructed in terms of their complete CDS sequences. *ZmmICE1* showed high homology with maize, rice, wheat and *Brachypodium distachyon*. These results indicated that ZmmICE1 has typical features of ICE-like proteins and is closely related to some homologues in monocots (Figure 4.1B). The predicted three dimensional structures of the bHLH-ZIP domain of ZmmICE1 were distinct, and were more similar to the structures of fold library id: d1am9a (Figure 4.1C).

A semi-quantitative RT-PCR was used to examine the expression profiles of *ZmmICE1* in leaves of *Zea mays* ssp. *mexicana* L. over a cold stress time course (Figure 4.1D). *ZmmICE1* expression increased significantly after 3 h, followed by a gradual decline from 6 h to after 24 h. These results suggested that *ZmmICE1* may be involved in cold stress responses.

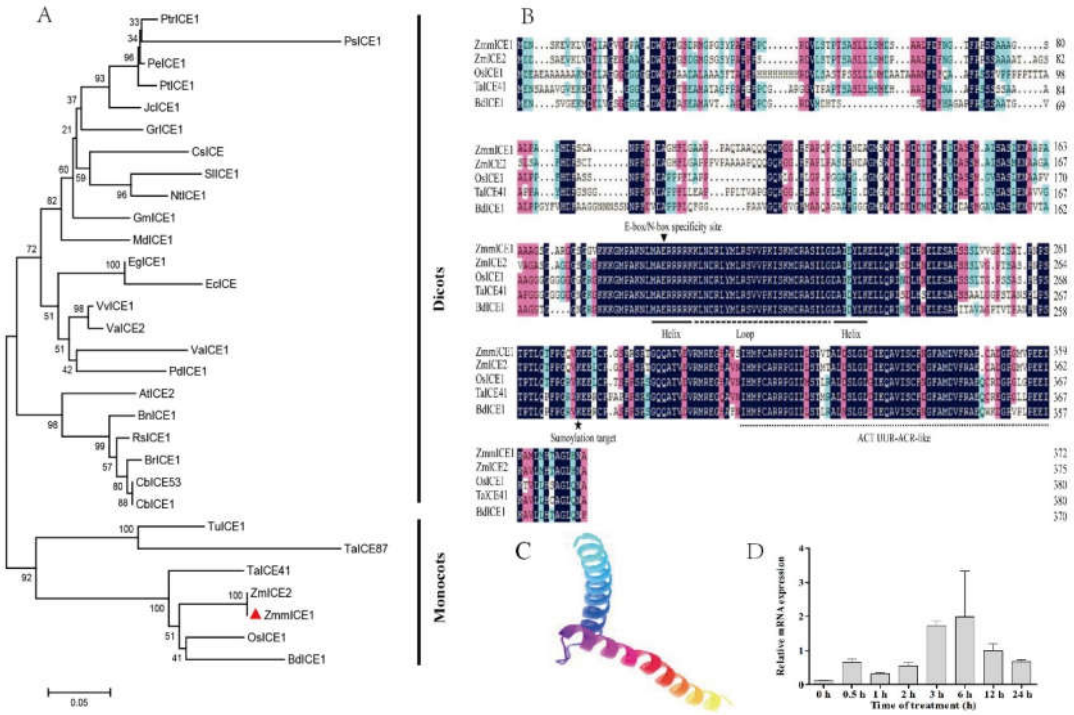

Figure 4.1 Multiple sequence alignment of ZmmICE1 with typical ICE-like proteins from other plants, structure analysis and expression profile of ZmmICE1.

*(A) Phylogenetic tree based on the deduced amino acid sequences of ICE from a range of plant species. The origin and GenBank accession numbers of compared sequences are as follows: *PtrICE1* [*Populus trichocarpa*] (ABN58427); *PsICE1* [*Populus suaveolens*] (ABF48720); *PeICE1* [*Populus euphratica*] (XP_011040262); *PtICE1* [*Populus trichocarpa*] (XP_002318166); *JcICE1* [Jatropha curcas] (NM_001319930); *GrICE1* [*Gossypium raimondii*] (XP_012489049); *CsICE* [*Camellia sinensis*] (ACT90640); *SlICE1* [*Solanum lycopersicum*] (NP_001274718); *NtICE1* [*Nicotiana tomentosiformis*] (XP_009625133); *GmICE1* [*Glycine max*] (NM_001251631); *MdICE1* [*Malus domestica (apple)*] (NM_001294038); *EgICE1* [*Eucalyptus globulus*] (AEF33833); *EcICE* [*Eucalyptus camaldulensis*] (ADY68776); *VvICE1* [*Vitis vinifera*] (AFI49627); *VaICE1,VaICE2* [*Vitis amurensis*] (AGP04217, AGP04218); *PdICE1* [*Prunus dulcis*] (ALI97583); *AtICE2* [*Arabidopsis thaliana*] (NP_172746); *BnICE1* [*Brassica napus*] (XP_013698429); *RsICE1* [*Raphanus sativus*] (ADY68771); *BrICE1* [*Brassica rapa* (field

mustard)] (XM_009153774); *CbICE1, CbICE53* [*Capsella bursa-pastoris*] (AAS79350, AY506804); *TuICE1* [*Triticum urartu*] (EMS66007); *TaICE41, TaICE87* [*Triticum aestivum*] (ACB69501, ACB69502); *ZmICE2* [*Zea mays*] (ACG46593); *OsICE1* [*Oryza sativa Japonica* Group] (BAD88163); *BdICE1* [*Brachypodium distachyon*] (XP_003567427), was produced by MEGA 5.1 with 500 bootstrap tests by Neighbor-joining tree. (B) Comparison of ZmmICE1 amino acid sequences with homologous proteins. ClustalX 1.8 and DNAman 8.0 alignment were employed for deduced sequence homologous alignment. The Sequences and accession numbers are shown for the following: ZmmICE1, ZmICE2, OsICE1, TaICE41 and BdICE1 as described previously. Residues in black and gray regions indicate identical and similar residues, respectively, between isoforms. Two predicted conserved fragments are labeled: a basic-helix-loop-helix region and a C-terminal conserved ACT-UUR-ACR-like domain. The five-pointed star indicates the sumoylation target site. The triangle indicates the E-box/N-box specificity site. (C) The predicted tertiary structure of the bHLH-ZIP domains of the ZmmICE1 protein evaluated by phyre2 online server. (D) *ZmmICE1* gene expression pattern in *Zea mays* ssp. *mexicana* L. under abiotic stress. The relative transcript abundances of *ZmmICE1* under 4 °C treatments at the indicated time were determined via quantitative RT-PCR with 14-week-old plants. Data are the means +/- SE (n=3) of a representative data from one biological replicate.

4.3.2 Transcriptional activation of *ZmmICE1* in yeast

Yeast one hybrid assays were carried out to determine whether *ZmmICE1* possess transactivation activity. The results showed that pGBKT7-*ZmmICE1* + pGADT7 could not only grow well on the SD/-Leu/-Trp medium, but also grew normally on the SD/-Leu/-Trp/-His + 0.5 mM 3-AT medium, both exhibiting *β*-galactosidase activity. In contrast, transformants carrying the empty contrast (pGBKT7 + pGADT7) did not grow on either the SD/-Leu/-Trp/-His + 0.5 mM 3-AT medium or SD/-Leu/-Trp/-His + X-a-Gal medium,

showing no *β*-galactosidase activity. The results indicated that ZmmICE1 has transactivation activities in yeasts (Figure 4.2A).

4.3.3 Subcellular location and sumoylation assay of *ZmmICE1*

Sequence analysis showed that *ZmmICE1* had a similar structure to other *ICE*-like transcription factors, suggesting that this gene performs functions in the nucleus. In order to verify this, *ZmmICE1* was transiently expressed as translational fusions at the C terminus of YFP in protoplasts of *Arabidopsis*. Confocal imaging identified that YFP alone (control) resulted in a diffused distribution of yellow fluorescence throughout the entire protoplast. As suggested, cells transformed with YFP::*ZmmICE1* showed strong fluorescence exclusively in the nucleus (Figure 4.2B).

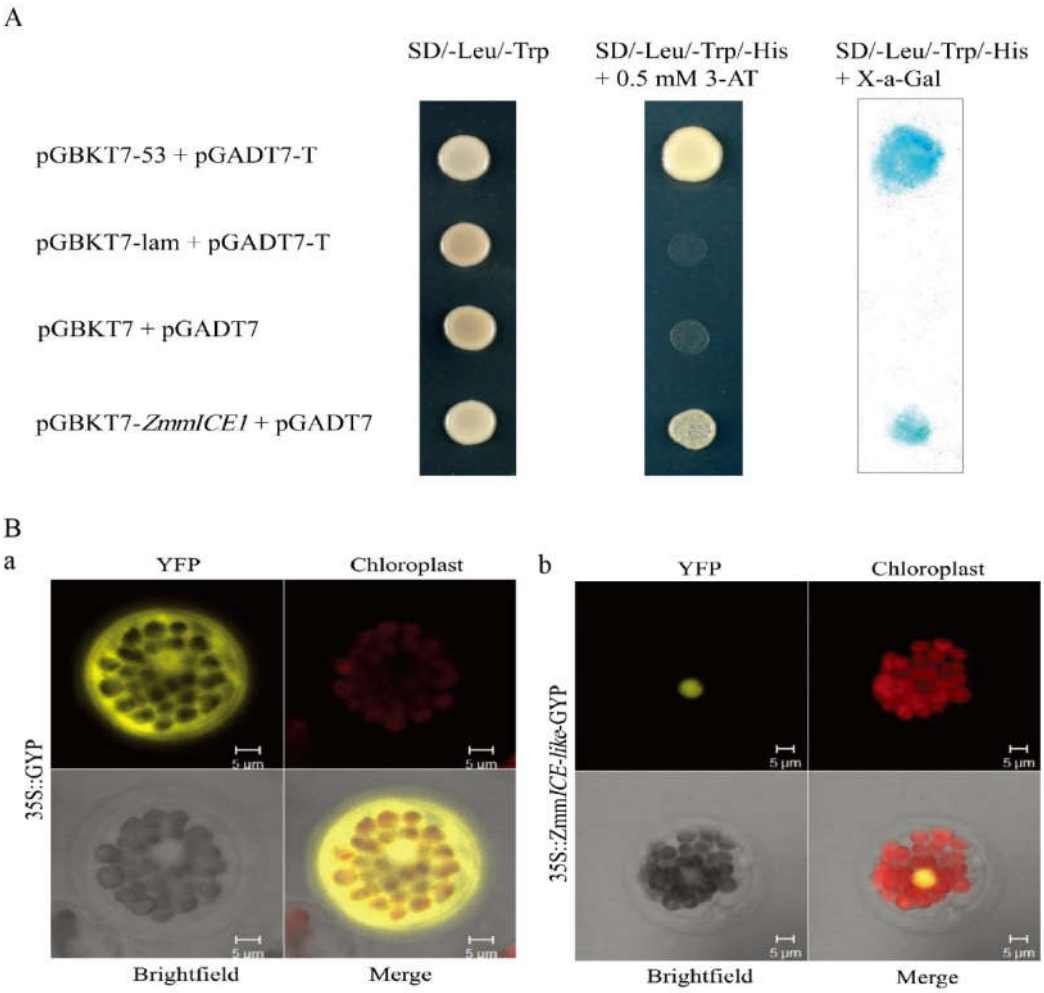

Figure 4.2 Transcriptional activation assay of ZmmICE1 and subcellular localization of ZmmICE1 protein in protoplasts of *Arabidopsis thaliana*.

*(A) Fusion proteins of pGBKT7-*ZmmICE1*, pGBKT7-53 + pGADT7-T (positive control), pGBKT7-lam + pGADT7-T (negative control) and pGBKT7 + pGADT7 (empty contrast) were expressed in the yeast strain AH109. Transformants were incubated on both SD/-Leu/-Trp, SD/-Leu/-Trp/-His/0.5 mM 3-AT and SD/-Trp/-Ade/-His/X-a-gal media to assess their growth and test *β*-galactosidase activity. (B) Subcellular distribution of YFP-fused ZmmICE1 protein in wild-type protoplasts, driven by the 35S promoter. YFP alone was used as a control. The images include dark field images of yellow fluorescence of the YFP, chloroplast autofluorescence, the morphology of protoplasts and the merged images. Representative YFP signals as

shown in from the majority of the observed protoplasts are shown.

In order to detect the sumoylation of ZmmICE1, we performed sumoylation assays in an *E.coli* strain expressing *ZmmICE1* together with AtSAE1a-SAE2, AtSCE1a and AtSUMO1 (Liu *et al.* 2015; Zhang, Qi & Yang 2010). The results showed that SUMO small molecules can covalently bind to the target gene when Sumo E1 and Sumo E2 exist. Thus, *ZmmICE1* has sumoylation specific binding sites, which is in accordance with its *ICE*-like features and bioinformatical predicted outcomes (Figure 4.3).

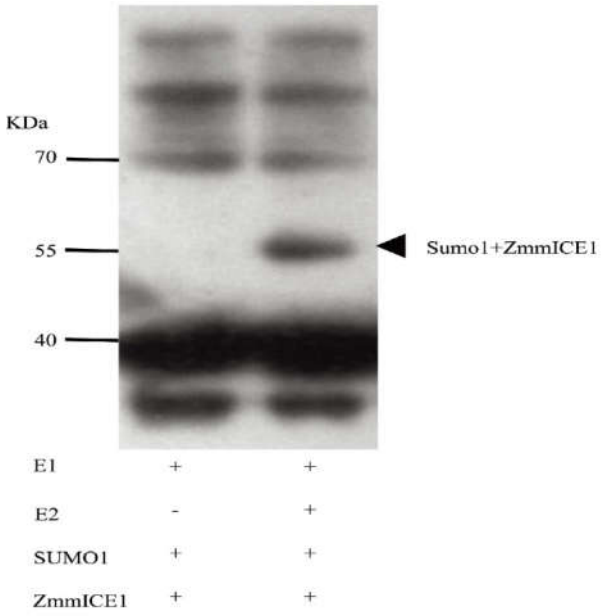

Figure 4.3 ZmmICE1 sumoylation *in vitro* assay result.

*pCDFDuet-1-Flag-ZmmICE1 were expressed in *E. coli* and then tested for SUMOylation activity. Immunoblots generated from these samples were probed with anti-Flag antibodies.

4.3.4 Transgenic *Arabidopsis* showed better freezing tolerance

In order to examine the function of *ZmmICE1* in freezing tolerance response, *Arabidopsis ice1-2* mutants were transformed with constructs carrying *ZmmICE1*. Transgenic and control (wild type) plants showed no obvious differences (Supplemental file 8). Transgenic CM 22:22 and CM 22:27 lines with controls were subjected to a whole plant freezing assay to investigate plant survival after freezing treatment (Figure 4.4). The freezing assay was

performed with three-week-old transgenic and control seedling plants treated with temperatures of -6 °C for 8 h and then recovery to normal conditions for 7 d. The *ZmmICE1* transgenic plants showed better cold tolerance and recovery capability. These results suggested that *ZmmICE1* improved freezing tolerance of *Arabidopsis*.

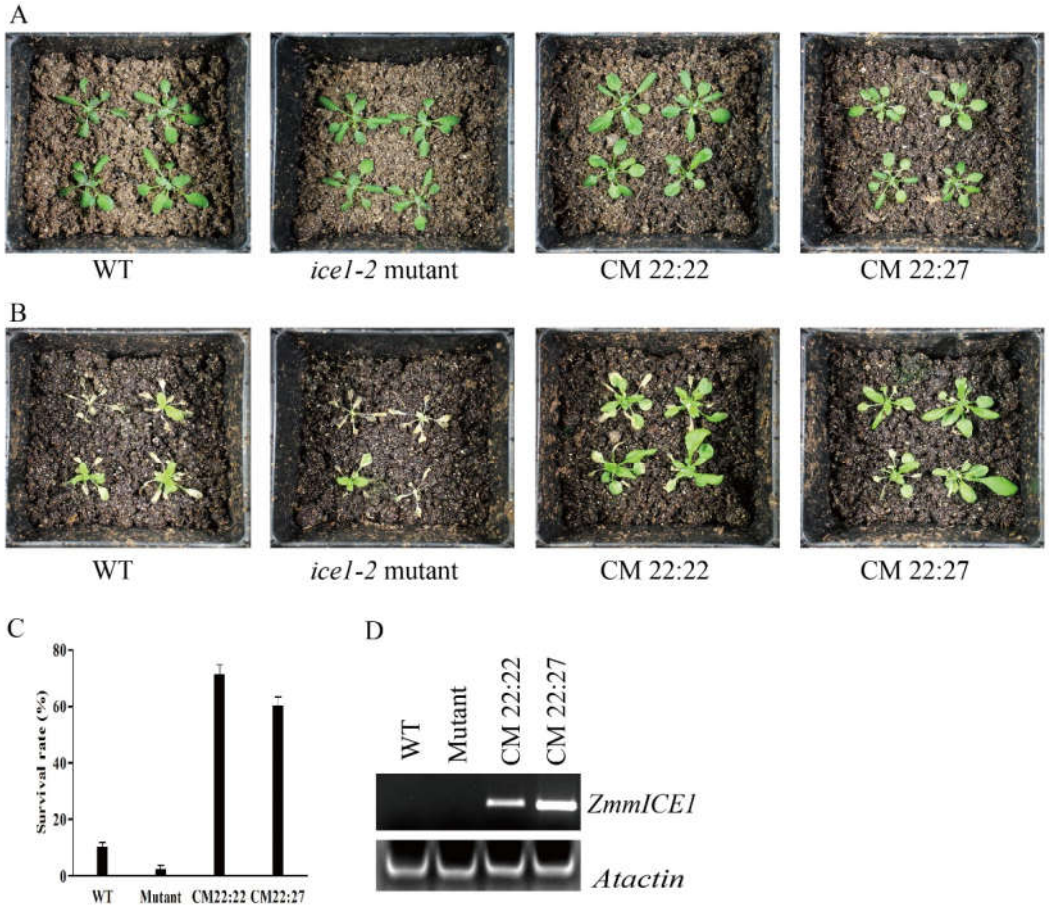

Figure 4.4 Freezing tolerance evaluation of ZmmICE1 transgenic *Arabidopsis* plants. *Three-week-old plants were treated at -6 °C for 8 h and then transferred back to normal conditions for recovery. Photographs were taken at -6 °C for 8 h (A) after 7 d of recovery (B). (C) Average survival rates and standard errors were calculated using the results of three separate experiments with 50 seedlings per line for each freezing stress. (D) RT-PCR was used to assess the transcript abundance of ZmmICE1 in the transgenic *Arabidopsis* plants. *Atactin* was used as a reference control. WT=wild type, *ice1-2* mutant and transgenic lines CM 22:22/7.

4.3.5 Transgenic plants showed electrolyte leakage as well as increased proline and malondialdehyde

The major stress physiological indexes: proline, MDA and EL are commonly used to evaluate oxidation stress and membrane injury with cold stress (Campos *et al.* 2003; Hadi, Gilpin & Fuller 2011; Jin *et al.* 2016). An experiment was conducted to compare levels of these indicators in three-week-old, previously non-acclimated WT, *ice1-2* mutant and *ZmmICE1* overexpressed plants (CM 22:22, CM22:27) which had been treated using -2 °C freezing conditions (Supplemental file 10). Under non-acclimated conditions, EL values were basically the same in the transgenic plants (between 11.35% and 19.35%) for the first 12 hours whereas there were significant rises in the WT and mutant plants within 3 hours (Figure 4.5A). When the stress time was extended to 48 h, the EL of WT and mutant plants had increased significantly by 85% to 89%. However, transgenic lines only had an increase of 51.65% for CM 22:22 and 63.29% for CM 22:27 (Figure 4.5A). The proline contents of control plants of all genotypes were similar, ranging from 38.06 μg/g to 43.31 μg/g. -2 °C freezing treatment showed little effect on proline contents of all genotypes (Figure 4.5B). MDA contents of all plants had similar start levels before the freezing treatment, ranging from 3.2 μg/g to 4.7 μg/g. However, MDA accumulation of both WT and the mutant increased gradually with prolonged time of freezing treatment and reached 14.22 μg/g and 18.32 μg/g at 48 h, respectively (Figure 4.5C). In contrast, freezing treatment showed no significant effect on MDA accumulation in *ZmmICE1* transgenic plants.

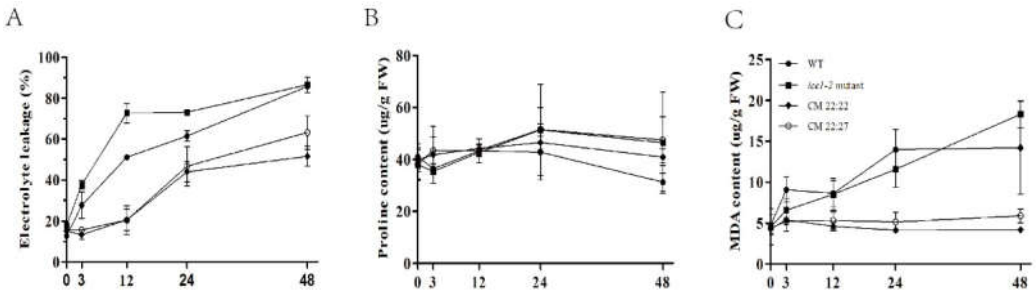

Figure 4.5 Effect of ZmmICE1 expression in *Arabidopsis* on levels of (A) electrolyte

leakage, (B) proline and (C) malondialdehyde (MDA).

*Three-week-old *Arabidopsis* plants of wild type, *ice1-2* mutant and transgenic lines CM 22:22 and CM 22:27 were grown at -2 °C for the time indicated. Leaves were collected to assess electrolyte leakage and free proline and MDA. Each value is the mean ± SD of three replicates and mixed sampling for the indicated time points.

4.3.6 *ZmmICE1* positively regulates the expression of *CBFs* and downstream target genes

In order to identify the relationship between *ZmmICE1* and the molecular components of the ICE-CBF pathway, we investigated the expression patterns of freezing tolerance genes of the 35S::*ZmmICE1* complementary transgenic *Arabidopsis* lines CM 22:22 and CM 22:27 using real-time PCR (Figure 4.6). Under normal conditions, the expression of identified genes was relatively low in both control and transgenic plants. However, all of the *CBFs* family genes, including *AtCBF1*, *AtCBF2*, *AtCBF3,* were up-regulated significantly in transgenic plants after freezing stress for 12 h compared to control plants. The expression of these three genes were reduced to very low levels 48 h after freezing treatment. The expression of *CBF* response downstream genes, i.e. *AtCOR15A*, *AtCOR47*, *AtKIN1* and *AtRD29A*, was also investigated. All four genes were up-regulated for both transgenic genotypes at 12 h after freezing treatment. The levels of the expression of all four genes in CM 22:22 were reduced at 48 h after freezing treatment though they still remained above initial values. For CM 22:27, there was unchanged *AtCOR15A*, *AtCOR47* expression, increased *AtKIN1* expression and reduced (though still above initial value) *AtRD29A* expression at 48 h of freezing treatment. These results suggested that *ZmmICE1* positively regulates the expression of the *CBF* genes and downstream target genes in response to freezing stress and may thereby improve cold tolerance.

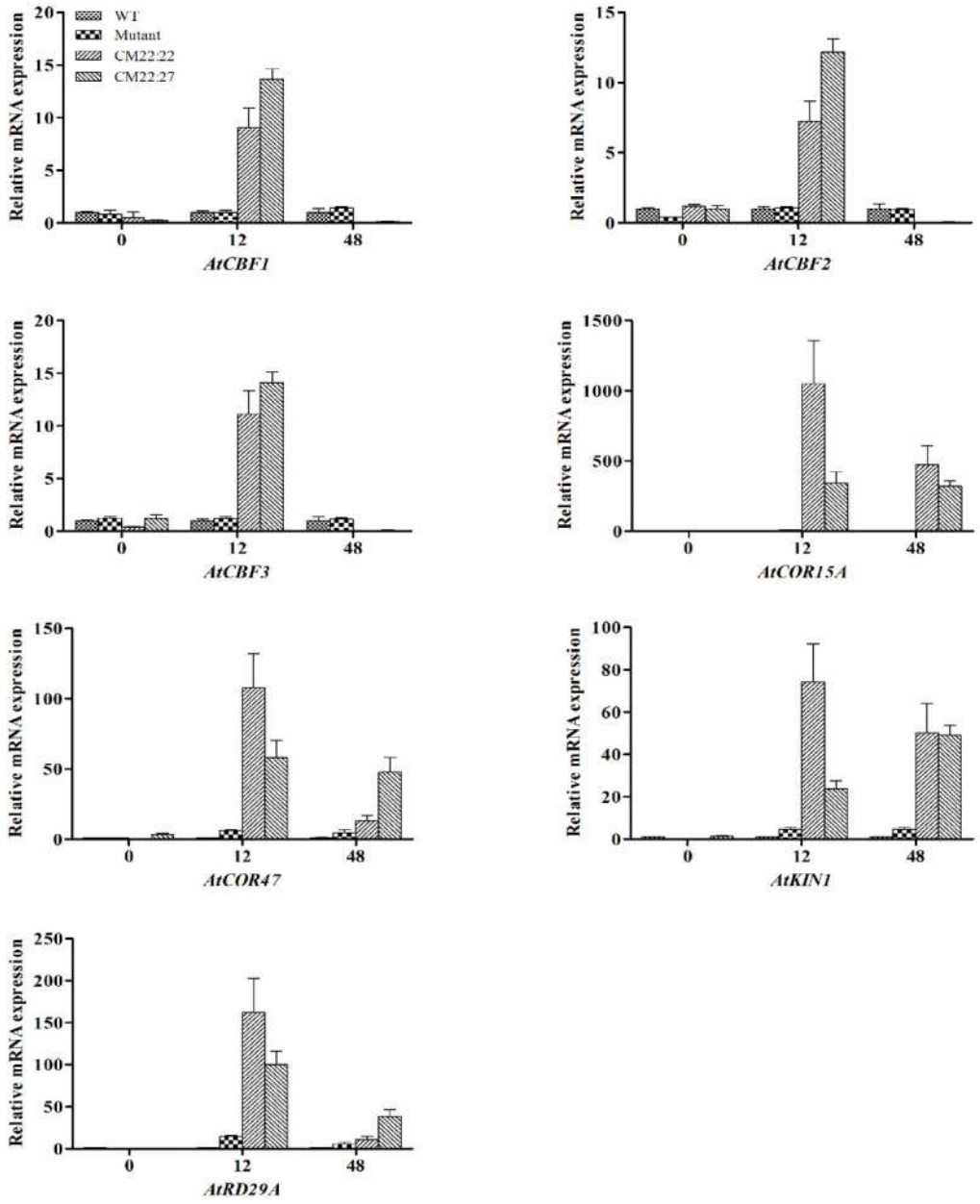

Figure 4.6 Transgenic *Arabidopsis* with ZmmICE1 on transcript levels of genes involved in the cold stress pathway.

*qRT-PCR analysis using leaves from wild type and *ice1-2* mutant control and transgenic lines CM 22:22 and CM 22:27 *Arabidopsis* plants. Three-week-old plants were grown at 0 °C for the time indicated. The tested genes were *AtCBF1, AtCBF2, AtCBF3, AtCOR15A, AtCOR47, AtKIN1* and *AtRD29A*. *β-tubulin* was used as a reference control. Each value is

the mean of three replicates.

4.4 Discussion

Zea mays ssp. *mexicana* L., a member of the teosintes, is a tropical plant and a wild relative of maize. Similar to maize, it is considered a cold-sensitive species with a relatively high temperature threshold for germination and vegetative growth (Cohen & Galinat 1984; Reeves 1950). In this study, the cold related *ZmmICE1* gene was isolated from *Zea mays* ssp. *mexicana* L. *ZmmICE1* contains a highly similar conserved basic helix-loop-helix (bHLH) domain and C-terminal region of ICE-like proteins based on sequence analysis and shows transactivation activities in yeast as *SlICE1* (Feng *et al.* 2013) . The bHLH TFs represent a family of proteins that contain a conserved bHLH domain, a motif involved in DNA binding, and regulate downstream genes through sequence-specific interactions in the promoter regions (Feng *et al.* 2012). Many different types of *ICE*-like genes with similarly conserved domain structure have been detected and cloned in different higher plants. These genes include *AtICE1, AtICE2, ValICE1, ValICE2, EcalICE1, SlICE1, TaICE141, TaICE187, ItICE1, CsICE1* (Badawi *et al.* 2008a; Dong *et al.* 2013; Fursova, Pogorelko & Tarasov 2009; Lin *et al.* 2014; Miura *et al.* 2012; Wang *et al.* 2012; Xiang *et al.* 2013). The *ZmmICE1* sequence showed a lack of the typical S-rich motif of ICE protein in common with *TaICE41* [*Triticum aestivum*] (ACB69501); *ZmICE2* [*Zea mays*] (ACG46593); *OsICE1* [*Oryza sativa Japonica* Group] (BAD88163) and *BdICE1* [*Brachypodium distachyon*] (XP_003567427) which all belong to the monocot group with many similar common traits. However, the S-rich domain is an important domain for part of dicots *ICE*-like genes such as *VaICE1* [*Vitis amurensis*] (AGP04217), *AtICE1, AtICE2* [*Arabidopsis thaliana*] (AT3G26744, NP_172746), *CbICE53* [*Capsella bursa-pastoris*] (AY506804), *SlICE1*, *SlICEa* [*Solanum lycopersicum*] (AK247172, JX625139), *GmICE1* [*Glycine max*] (FJ393223), *PsICE2* [*Populus suaveolens*] (HM627255) (Badawi *et al.* 2008b; Feng *et al.* 2013; Wang *et al.* 2012; Xu *et al.* 2014a) (Supplemental file 9). Therefore, we predicted that S-rich structure is a possible feature of *ICE*-like between part

of monocots and dicots plants. Our results suggested that *ZmmICE1* is a putative *Zea mays* ortholog of the ICE proteins. As an important post-translational modification process, sumoylation modification in plants has been identified in numerous basic cellular processes, such as stress and defense responses and growth development (Miura & Hasegawa 2008; Miura *et al.* 2005; Zhang *et al.* 2013a). Different *ICE*-like genes in various plants have different posttranslational modification results. For example, *Vitis* ICE2 and ICE3 contain putative sumoylation and ubiquitination motifs, suggesting that ubiquitination and sumoylation processes are involved in regulating expression activity. AtICE1 sumoylation has also been identified in *Arabidopsis* (Thomashow 2010). In our study, the sumoylation of ZmmICE1 was identified by *in vitro* experiment, suggesting that Sumo E1 and Sumo E2 are enough for the sumoylation of substrate without E3 ligase in *Zea mays* ssp. *mexicana* L. Thus, *ZmmICE1* could be a putative *Zea mays* gene ortholog of the ICE protein genes.

Cold temperature stress causes changes in physiological and biochemical metabolic substances, the major stress physiological indexes: proline, MDA and EL are commonly used to evaluate oxidability and membrane integrity (Campos *et al.* 2003; Hadi, Gilpin & Fuller 2011; Jin *et al.* 2016; Rudolph & Crowe 1985; Zhang *et al.* 2010). Overexpression *Vitis amurensis ICE1* and *ICE2* in *Arabidopsis* can improve cold tolerance and simultaneously change the contents of EL, proline and MDA as well as survival rates (Xu *et al.* 2014a). Similarly, overexpression of *Chrysanthemum dichrum ICE1* in *Arabidopsis* has been shown to reduce EL level and improve freezing tolerance (Chen *et al.* 2013). In our study, survival rates of transgenic plants improved markedly after freezing treatment. The content of EL and MDA of wild type and mutant plants increased quicker than transgenic plants, indicating increased freezing tolerance in transgenic plants. Dramatic accumulation of proline due to increased synthesis and decreased degradation under a variety of stress conditions, which are oppositely regulated by the proline biosynthesis key enzyme catalyzing gene P5CS (d-1-pyrroline-5-carboxylate synthetase) and ProHD (proline dehydrogenase), have been reported (Ibragimova *et al.* 2012; Kaur & Asthir 2015;

Moiseeva *et al.* 2012). However, no significant changes in proline contents were observed in our experiment.

Under cold temperature stress, a plant signal transduction pathway is activated, which includes a series of molecular response mechanisms. The most important cold acclimation signal regulatory pathway is the ICE1-CBF-COR transcriptional cascade (Chinnusamy, Zhu & Zhu 2007; Lissarre *et al.* 2010; Zhu *et al.* 2007). The MYC-type transcription factor *ICE*-like is the most important member of the cold signaling response cascade pathway, which induces downstream *CBF* transcription factors and further combines with CRT/DRE cis-elements (A/GCCGAC) of the promoter of *COR* genes to regulate the expression of *COR* genes in plants under cold stress (Chinnusamy *et al.* 2003; Miura & Furumoto 2013; Shi, Ding & Yang 2015). The transcription factor *ice1* mutant plant of *Arabidopsis* is sensitive to both chilling and freezing stresses, inducing the expression of *CBF3/DREB1A* and its regulons during cold acclimation (Lee, Henderson & Zhu 2005). Over-expression of *AtICE1* increased the expression of *AtCBF2*, *AtCBF3* and cold-regulated genes under cold stress (Barrett *et al.* 2004). In addition, *ICE2*, a homolog gene of *ICE1* positively regulates *CBF1* expression and enhances plant freezing tolerance (Fursova, Pogorelko & Tarasov 2009; Kurbidaeva, Ezhova & Novokreshchenova 2014). In our study, *AtCBF1*, *AtCBF2* and *AtCBF3* all had high expression in both transgenic lines at 12 h after freezing treatment, indicating that *ZmmICE1* induced *CBF* genes expression. *ZmmICE1* and *Arabidopsis ICE1*/*2* cDNA protein sequences are not highly homologous (36.22–39.82%) and *AtICE1* and *AtICE2* are functionally redundant in freezing tolerance, thus it is suggested that *ZmmICE1* plays an important role not only in *AtCBF2* and *AtCBF3* expression, but also in *AtCBF1* expression. *COR* plays important roles in chilling tolerance and cold acclimation with nearly 40% of *COR* genes and 46% of cold-regulated transcription factor genes being regulated by *ICE1* (Lee, Henderson & Zhu 2005). Overexpression of *COR* genes can significantly improve plant cold tolerance. In *Arabidopsis*, group 2 LEA (LEA II) proteins, *KIN* and *CORs* genes such as *COR78/RD29A,*

COR47, COR15, COR6.6, COR413 and other dehydrins are induced by cold stress (Hundertmark & Hincha 2008; Shao, Liang & Shao 2005). Our results showed that most of *CBF* downstream target genes were up-regulated from 12 h to 48 h after freezing treatment in *ZmICE1* transgenic plants. In addition, the expression level of CBF1 and CBF3 did not elevate (even lower than that of WT and ice1-2) in ICE1 overexpression lines at time point 0 h. The induction of CBF-COR were only observed at 12 h and 48 h in overexpressing lines, suggesting that under non-cold stress, overexpression of ICE1 has less effect on induction of CBF-COR, while under cold stress, the over-expression lines obviously induced CBF-COR. It is possible that co-suppression of the endogenous gene occurred in the target plant under non-cold stress, but we has mentioned that the cDNA nucleotide sequences are not highly homologous between *ZmmICE1* and *AtICE1/2*. Under cold stress, the ZmmICE1 are abundant in the transgenic lines at mRNA level, which caused the response of downstream genes. These results suggested that *ZmmICE1, a* homolog of *Arabidopsis* AtICE1/2, plays important roles in the regulation of freezing stress response.

4.5 Conclusion

In conclusion, we identified a MYC-type *ICE*-like TF that encodes a bHLH transcription factor named as *ZmmICE1*, from *Zea mays* ssp. *mexicana* L. *ZmmICE1* is localized in the nucleus and participates in a sumoylation modification process. An assay for *ZmmICE1* complementary overexpression showed improved freezing tolerance of transgenic *Arabidopsis thaliana ice1-2* mutant. These results have provided additional evidence for *ICE* family genes functioning in low temperature tolerance and substantially increase the understanding of the molecular basis of cold tolerance mechanisms. It can also offer better reference information for understanding teosinte and other crop cold tolerance mechanisms.

Chapter 5 Seedling photosynthesis and chlorophyll fluorescence responses of varieties of *Zea mays* ssp. *mexicana* L. with different induced chilling tolerances

Abstract

The closest wild relative of maize, the annual *Zea mays* ssp. *mexicana* L., a member of teosinte, has strong growth and regeneration ability, high tiller numbers, high protein and lysine content as well as resistance to many fungal diseases. It is a tropical crop with low tolerance to low temperature environments. We have used chlorophyll (Chl) *a* fluorescence to evaluate the low temperature response of differential varieties of *Zea mays* ssp. *mexicana* L. seedlings plants, "8493", "You12" and an "Inbred line" constructed by South China Normal University (SCNU). Plants were grown under three temperature conditions, 25 ºC as control, 15 ºC and 5 ºC as treatments. The ChlF parameters: Non-photochemical quenching (NPQ), the maximum photochemical efficiency of photosystem II (F_v/F_m), quantum efficiency of excitation energy trapping of PSII (F_v'/F_m'), effective quantum efficiency of PSII photochemistry (ΦPSII), Photochemical quenching (qP) and Electron transport rate (ETR) were measured as they are important indicators to evaluate cold stress in terms of severity, and the photosynthetic processes affected. In addition, chlorophyll contents (Chl a/b) were measured as an auxiliary method for helping to understand the chilling performance of differential varieties. The results showed that the *Fv/Fm*, *Fv'/Fm'*, ΦPSII, qP, NPQ and ETR of the 3 varieties were all lower under chilling conditions when compared to the control at 25 ºC, differences were significant at 5 ºC , and with increasing times of treatments. The results clearly demonstrated the presence of cold tolerance differences among the 3 varieties of *Zea mays* ssp. *mexicana* L., and quantified the degree of damage to the PSII response with chilling stress of *Zea mays* ssp. *mexicana* L. Overall "Inbred line" was shown to be more sensitive to chilling stress than the other varieties.

Key words: *Zea mays* ssp. *mexicana* L., chlorophyll fluorescence, chilling tolerance, seedling stage

5.1 Introduction

Most major crops are cultivated away from their original area and climate zones. In order to survive and harvest yield in nature, plants require effective adaptability and resistance development to adapt to changes in their environment. Through the evolution of genetic type to develop changes in the plant's physiology and shifts in its ecological niche resistance can be developed. Low temperature stress is a major environmental factor that deeply impacts on the geographical distribution and species composition of plant communities. It can also lead to a decrease of crop quality and productivity (Puyaubert & Baudouin 2014; Viswanathan & Zhu 2002). Many temperate regions crops are very sensitive to low temperature if the plants have not experienced effective low temperature acclimation (Venema, Villerius & van Hasselt 2000). In general, compared with mature plants, the seedling growth stage is more sensitive to chilling stress and chilling injury leads to plant loss of viability. Moreover, flower organs will be more seriously influenced by chilling stress at the reproductive growth stage (Sayed 2003b). With chilling injury between 0 to 15 °C, leaf metabolism is greatly inhibited and recovers slowly, which is caused by the photo damage to the PSII reaction center protein D1 (Baker & Rosenqvist 2004; Kyle, Ohad & Arntzen 1984). The intensity of the stress applied has a significant influence on the recovery rate: the higher the stress, the slower the recovery rate of PSII. In laboratory studies, young maize seedlings have been studied using cold room treatments (< 5 °C) for a short period. These have clearly demonstrated that cold impairs the photosynthetic machinery and suggested the physiological mechanisms of cold tolerance (Riva-Roveda *et al.* 2016). In addition, experiments have identified that growth of maize is seriously limited below 15 °C and the effective chilling range is between 15 °C and 5 °C, which leads to reduced leaf growth between these temperatures. The impaired photosynthesis consequently caused reduced plant productivity in terms of biomass or

grain yield (Theocharis, Clément & Barka 2012).

Maize genetic resources from the Mexican highlands are known to be a potential source for improved chilling tolerance (Leipner 2009). The annual *Zea mays* ssp. *mexicana* L. (*Zea mexicana*), a member of the teosinte, has strong growth and regeneration ability, high tiller numbers, high protein and lysine content as well as resistance to many fungal diseases (De Wet, Harlan & Grant 1971). It is a close wild relative of maize thus it can be effectively used for investigating maize genetics, genome evolution and breeding. Similar to maize, *Zea mexicana* is found at high altitudes in northern and central Mexico (about 1600~2700 m). It has a large spikeleted trait and the plants can adapt to acid soil and the best growing temperatures range from 25 ºC to 35 ºC (Fukunaga *et al.* 2005; Takahashi *et al.* 1999). It was considered to be a very cold-sensitive crop especially during germination and early autotrophic growth stages, despite its origin at altitudes of about 1,000-2,000 m (Bhosale *et al.* 2007; Hincha & Zuther 2014; Marocco, Lorenzoni & Fracheboud 2005; Miedema 1982). Photosynthesis is strongly affected due to the inhibition of certain enzymes of the C4 and the Calvin cycle. Temperatures below 10 ºC will delay or impede the morphological establishment and yields (Fukunaga *et al.* 2005; Wllkes 1977). The most apparent response of maize seedlings exposed to low temperature is the reduction of photosynthesis, especially the photosynthetic light reaction, which offers essential energy for life of photosynthetic organisms (Mstirling, Lrodrigo & Emberru 1993; Niazi *et al.* 2015b). In order to use *Zea mexicana* in a large-scale forage industry, low temperature and freezing tolerance are important traits for teosinte breeding, as they would allow for a longer growth period and more yield. Until now, the studies of *Zea mexicana* have mainly focused on genetic diversity, evolution and hybridization with cultivated maize (Almeida *et al.* 2011; De Wet, Harlan & Grant 1971; Fukunaga *et al.* 2005; Niazi *et al.* 2015a). As an important wild germplasm resources, extensive studies are still lacking for *Zea mexicana*, especially for abiotic stress effects. Available bolting-resistant, high yielding varieties with cold tolerance have not been fully exploited by the limited usage of wild

resources.

Chlorophyll fluorescence (ChlF) parameters have been identified as useful tools for evaluating low temperature response of oats, wheat and *Arabidopsis thaliana* plants (Fracheboud *et al.* 1999; Mishra, Heyer & Mishra 2014; Shangguan, Shao & Dyckmans 2000). In maize studies, the changes in ChlF signals have help detect, improve and understand cold induced response mechanisms, heavy metal toxicity, nitrogen fertilization and water-limited or drought condition responses in maize studies (Adam & Murthy 2014; Akram *et al.* 2011; Cai *et al.* 2012; Da Silva *et al.* 2012; Gouveia-Neto *et al.* 2013; Gouveia-Neto *et al.* 2012; Marques & do Nascimento 2013; Rodríguez *et al.* 2014; Rodríguez *et al.* 2013; Šimić *et al.* 2014; Wu *et al.* 2013). In addition, ChlF has been extensively used in large scale field and greenhouse industries as a highly efficient, inexpensive and rapid monitoring technique. However, the cold tolerance trait of *Zea mexicana* has rarely been studied and reported. Harvest data can be used to detect genotypes with improved seedling growth at low temperature which can be then screened against ChlF responses to compare reliability of the two methods.

In this study, we use non-destructive ChlF induction kinetics and ChlF imaging technology to evaluate the cold tolerance of varieties of *Zea mexicana* which earlier studies have indicated have differential responses to chilling because this ability has seldom been studied and reported previously (Fracheboud *et al.* 1999). Chl a fluorescence has been regarded as an effective tool to noninvasively monitor photosynthetic activity in vivo and has been extensively used to assess plant responses to differential types of abiotic stresses (Baker 2008; Kooten & Snel 1990). Non-photochemical quenching (NPQ) and the maximum photochemical efficiency of photosystem II (F_v / F_m), effective quantum efficiency of PSII photochemistry (ΦPSII), F_q'/F_m' and F_v'/F_m' also have potential for use in screening to identify tolerance to high temperatures (Baker & Rosenqvist 2004; Sayed 2003b). The main aim of this experiment is to better study cold tolerance of *Zea mexicana* and offer better genetic reference information for improving chilling breeding.

5.2 Materials and methods

5.2.1 Plant materials and growth conditions

All the experiments were carried out using plants of the *Zea mexicana* variety "8493", "You12" and "Inbred line". "8493" and "You12" were commercial resources, which were introduced, improved and produced in Henan province (Wang 2005) and Guangxi province (Huang *et al.* 1992) in China, respectively. "Inbred line" was produced in Guangdong province. Thus among the lines are adaptations to multiple environments. These seeds were obtained from the School of Life Science of South China Normal University (SCNU, Guangzhou, China). Selected plump seeds were washed 3 times with distilled water, then soaked in 75% ethanol and 2% sodium hypochlorite for 10 min and 3 min, respectively, and washed 3 times with distilled water.

Seeds were soaked in 1000 ppm GA_3 solution for 24 hours at 30 ºC to improve germination in accordance with the operation manual from the International Center for Improvement of Maize and Wheat (CIMMYT) (Mondrus 1981; Taba *et al.* 2004a). Then the seeds were planted in plastic boxes (54 cm length, 28 cm width and 7 cm height) with soil substrates (Jiffy, Netherlands, http://www.jiffygroup.com/en/substrates/). The boxes were placed in a climate control box (RXZ 500-C, JIANGNAN Instrument) at 31 ºC under a 10-h light (50 $\mu m^{-2} s^{-1}$) / 28 ºC 14-h dark photoperiod, at a relative humidity of 60 %. The plants were watered and fertilized with compound fertilizer nutrient solution at 2 g/L as required (YaraMila^{TM}, Norway).

5.2.2 Seedlings cold treatment implementation

18 day old seedlings were used for the treatments. All plants were maintained under similar conditions except for temperature. The control group were grown with normal conditions at 25 ºC and treatment groups were transferred to climate control box (GXZ-0450, JIANGNAN Instrument) at 15 ºC or 5 ºC for 12 h, 1 d (24 h), 2 d (48 h) and 3 d (72 h) and 4 d (96 h) under the same light conditions. The cold treatment experiments were repeated three times. All measurements were performed on the fully expanded first leaves.

5.2.3 ChlF parameters

The following ChlF parameters were measured or calculated: F_o (the minimum fluorescence in dark adapted state), F_o' (the minimum fluorescence in light-adapted state) (Oxborough & Baker 1997), F_v/F_m (the maximum quantum efficiency of PSII photochemistry in dark-adapted state), F_v'/F_m' (the maximum quantum efficiency of PSII photochemistry in light-adapted state), $F_v/(F_m-F_o)$ (the fraction of PSII centers that are capable of photochemistry), F_q'/F_m' (the operating quantum efficiency of PSII photochemistry), F_q'/F_v' (the PSII efficiency factor). With F_m (the maximum ChlF in darkadapted state), F_m' (the maximum ChlF in light-adapted state), F_v (the variable fluorescence in dark-adapted state (F_m-F_0)), F_v' (the variable fluorescence in light-adapted state (F_m'-F_o')), F_q' (the difference between F_m' and F' measured immediately before application of the saturation pulse used to measure F_m'). We also estimated the nonphotochemical quenching of the ChlF as NPQ = (F_m-F_m')/F_m (Bilger and Björkman 1990). NPQ was used instead of nonphotochemical quenching coefficient (qN), because NPQ is unaffected by changes in the rate constant of PSII photochemistry and this is in contrast with qN.

5.2.4 Chl a fluorescence measurements

For determination of the above parameters, ChlF was measured using a ChlF image system (*CFImage, Technologica*, UK). The measurement procedure lasted 1 h and a modified protocol according to J. Leipner (Leipner 2009) were programmed. After 30 min dark-adapted materials were measured for their change actinic. The PPFD (0 mol photons m^{-2}•s^{-1}) for detecting the minimum fluorescence F_o. F_m was measured with a saturation pulse (PPFD 6,321 μmol•m^{-2}•s^{-1}) of light for 800 ms plus length. After 5 s, the plants were exposed to a progressive increase of the actinic; then after 90 s, the plants were exposed to saturation pulse (PPFD 6,321 μmol•m^{-2}•s^{-1}) for the quenching analysis. The actinic of each cycle with PPFD 50, 100, 200, 400, 600, 800, 1000, 1200, 1500 μmol•m^{-2}•s^{-1}. Then use 3 minutes lasted apply pulse 2 cycles for detected steady states characters under light

adaption. 3 seedlings of each treatments were used to be measure. The transient traces can be recorded simultaneously. The ChlF were done on the middle parts of leaves. The ChlF parameters of the induction curve were automatically generated imaging system (Baker & Oxborough 2004).

5.2.5 Measurement of contents of total chlorophyll

Photosynthetic pigments were extracted from 0.2 g fresh leaf tissues which were soaked in a 10 mL mixture of solutions (80 % acetone + 95 % alcohol) 48 h to be extracted completely. The absorbances of the extracts were measured at 663 and 645 nm using an EnSpire multimode plate reader (PerkinElmer). The running program selected double orbital shaker mode of duration 30 s and speed of 300 rpm and diameter 0.3 mm. Measurement of 663/645 was made with a 7 mm measurement height and 10 flashes. The content of chlorophyll a, b and a+b were calculated using adjusted extinction coefficients (Lichtenthaler 1987). The Photosynthetic pigment content was expressed as $mg \bullet g^{-1}$ FW.

5.2.6 Statistical analysis

Image results were analyzed by one-way and LSD analysis of variance (*ANOVA*; significance level $p \leq 0.05$).

5.3 Results

5.3.1 Chl fluorescence characteristics

The maximal efficiency of PSII photochemistry (*Fv/Fm*) showed that the 3 varieties of *Zea mexicana* had significant changes with low temperature treatments at the seedling stage compared to the control. Cold stress of 5 ºC at the seedling stage significantly decreased *Fv/Fm.* The results showed that the range of *Fv/Fm* of the 3 varieties all fell in between 0.7-0.8 under 25 ºC with only small fluctuations appearing with 15 ºC stress. However, the value of *Fv/Fm* showed an obvious and significant downward trend among treatments as their severity increased under the 5 ºC conditions. ANOVA results showed that there were no significant differences between 25 ºC and 15 ºC, but there were significant differences

under 5 ºC stress at 96 h. At the 48 h treatment point, the 5 ºC treatment of "You12" reached 0.6 for *Fv/Fm*. The value of *Fv/Fm* showed some stabilization with longer time periods of chilling. The average *Fv/Fm* was reduced by 11 % relative to the controls under 5 ºC stress at 96 h, respectively (Figure 5.1, Supplemental file 11).

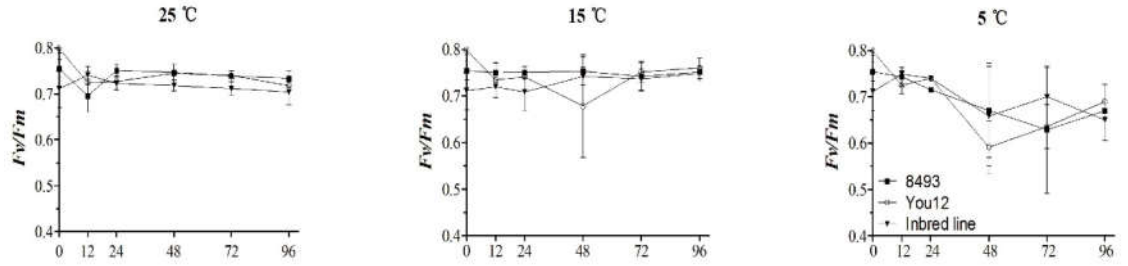

Figure 5.1 Chlorophyll fluorescence (ChlF) paratmters: maximum quantum yirld of PSII photosystems (F_v/F_m).

*The presented numeric values are mean of three independent plants with standard errors, and are integrated across the middle parts of leaves.

5.3.2 The light induction parameters

Quantum efficiency of excitation energy trapping of PSII (*Fv'/Fm'*) showed differential changes under differential treatment conditions. *Fv'/Fm'* showed lower values at 5 ºC than at 15 ºC or 25 ºC at 96 h, but there was no significant difference among lines at 96 h. Under 15 ºC stress, the downward trend with time of "Inbred line" was less than the other two lines (Figure 5.2, Supplemental file 12). However, according to the *ANOVA* results, only "You12" had significant changes under 5 ºC at 96 h.

The three lines had slightly different performances for quantum efficiency of the PSII open centers in the light adapted state (ΦPSII). At 25 ºC and 15 ºC there were no clear trends or significant differences. However, all lines declined at 5 ºC to approximately 0.1. The smallest decline was shown by "Inbred line". At the final test time point, "8493" and "You12" had stronger changes than "Inbred line", the average differences were 0.58, 0.37 and 0.31, respectively (Supplemental file 12). In addition, "8493" showed a faster decline

between 0 h to 24 h than the other lines, and showed a slight recovery from 24 h to 48 h (Figure 5.2).

With respect to photochemical quenching of the variable chlorophyll fluorescence (qP), the chilling stress at 15 ºC and 5 ºC lead to significant changes, which showed that all varieties had an obvious reducing trend (Supplemental file 12). Compared to 25 ºC, the average differences of "8493", "You12" and "Inbred line" under 15 ºC were 0.13, 0.13 and 0.18, respectively. In addition, "inbred line" showed the most significant changes under 15 ºC and 5 ºC when compared to the other varieties. The line "8493" had the lowest qP at 96 h under 5 ºC stress (Figure 5.2).

For non-photochemical quenching (NPQ), there were no clear and consistent significant differences among treatments (Supplemental file 12, Figure 5.2).

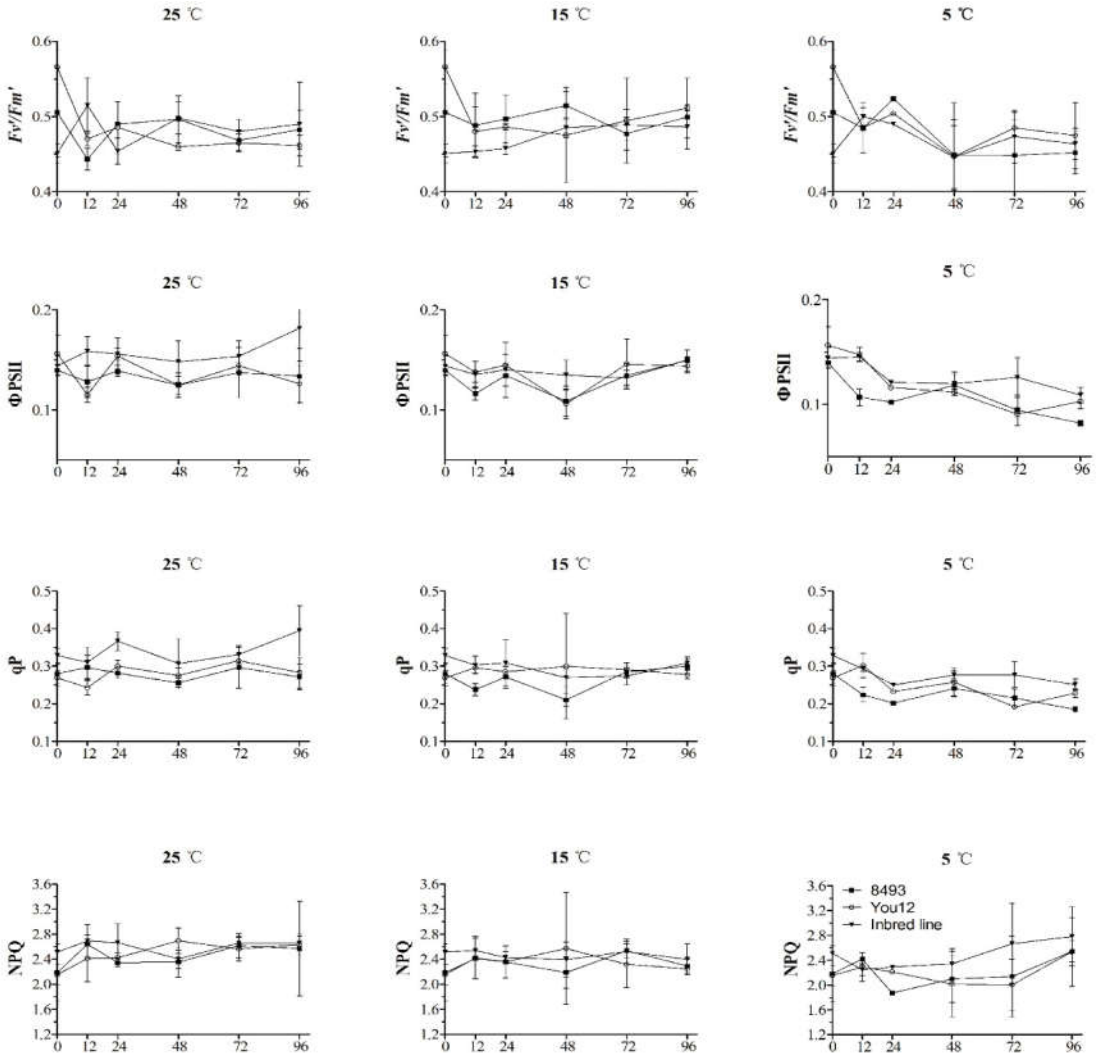

Figure 5.2 Chlorophyll fluorescence (ChlF) parameters: FV'/FM' quantum efficiency of excitation energy trapping of PSII of light-adapted leaves; ΦPSII efficiency of open reaction centre of light adapted state; qP photochemical quenching and NPQ nonphotochemical quenching.

*The error bars represent the standard error (±SE) for three replicates.

5.3.3 Fluorescence induction curve

The graphs for the induction curves showed that low temperature slightly increased the declined of the photochemical quenching (qP). Only "Inbred line" showed a significant

difference in the 15 ºC group. Varieties "8493" and "You12" have no obviously changes among temperature treatments (Supplemental file 13). The variety "Inbred line" had a higher qP than the other 2 varieties at 1500 μmol•m^{-2}•s^{-1}. However, "Inbred line" showed a slower decreasing trend than the other varieties. In particular, "Inbred line" have obvious differences from the others from 0 μmol•m^{-2}•s^{-1} to 400 μmol•m^{-2}•s^{-1} stage under 5 ºC stress (Figure 5.3).

The graphs for the induction curve of the nonphotochemical quenching (NPQ) showed that the NPQ of all varieties increased with increasing intensity of actinic light. When comparing the varieties all were similar at 25 ºC. However, at the lower temperatures "Inbred line" was not as affected with "8493" and "You12" showing lower slope. Only 5 ºC stress significantly affected the NPQ of "Inbred line (Supplemental file 13). There was no obvious difference between "8493" and "You12" (Figure 5.3).

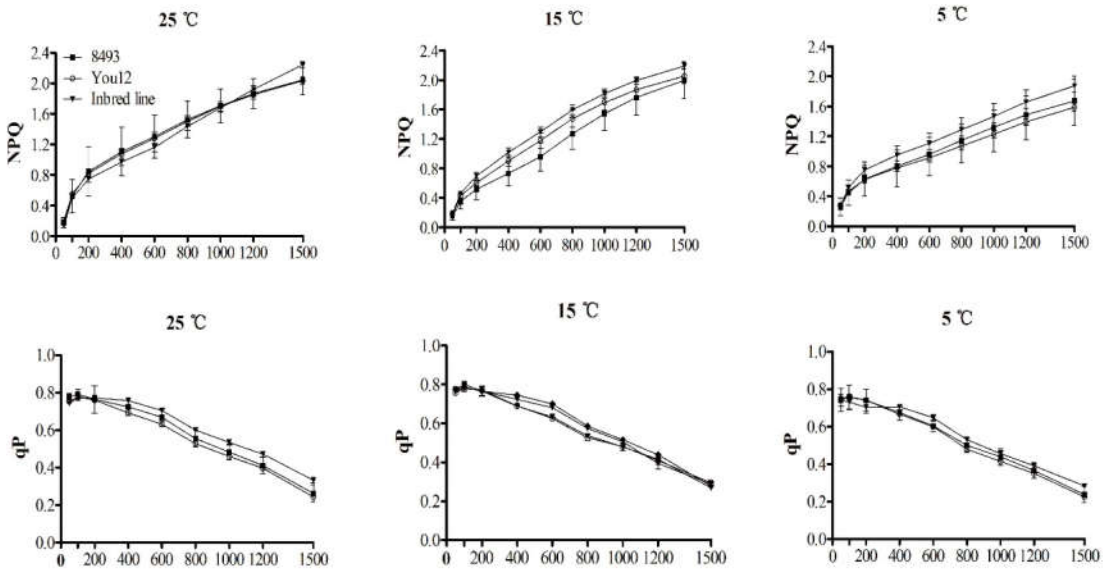

Figure 5.3 The light-response curve of the operating quantum efficiency of PSII photochemistry (qP), the maximum quantum efficiency of PSII photochemistry in light-adapted state (NPQ) for differential *Zea mays* ssp. *mexicana* L. plants grown and measured at 25 °C, grown at 25 °C and measured at 15 °C, grown at 25 °C and measured at 5 °C.

*The error bars represent the standard error (±SE) for three replicates.

5.3.4 ETR

The graphs for the induction curve of the nonphotochemical quenching (NPQ) showed that at the lower temperatures the peak for ETR declined in particular for "inbred line" (Supplemental file 14, Figure 5.4).

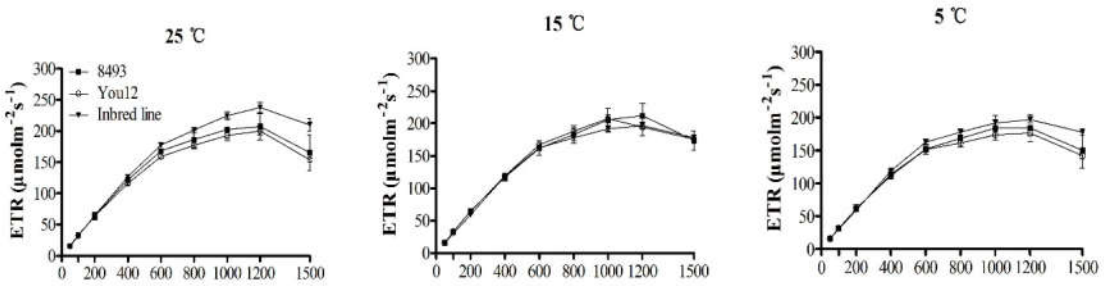

Figure 5.4 The light-response curve of the electron transport rate (ETR) for differential *Zea mays* ssp. *mexicana* L. plants grown and measured at 25 °C, grown at 25 °C and measured at 15 °C, grown at 25 °C and measured at 5 °C.

*The error bars represent the standard error (±SE) for three replicates.

5.3.5 Pigment analysis

The chlorophyll content (a/b, a+b) of all *Zea mexicana* cultivars had no significant changes with the 15 ºC treatment over time relative to the CK group. Taken across all treatment the inbred line has a lower overall chlorophyll a, b, and a+b content. However, the values of "8493" and "Inbred line" cultivars significantly increased with the 5 ºC low temperature treatment and 96 h stress (Supplemental file 15). The chlorophyll content (a, b, a+b) of "8493" had an increasing trend and reached 1.1 mg•g^{-1}, 0.4 mg•g^{-1} and 1.5 mg•g^{-1} for the 5 ºC treatment at 96 h. In addition, the "You12" showed the least changes with time at 96 h under 5 ºC (Figure 5.5).

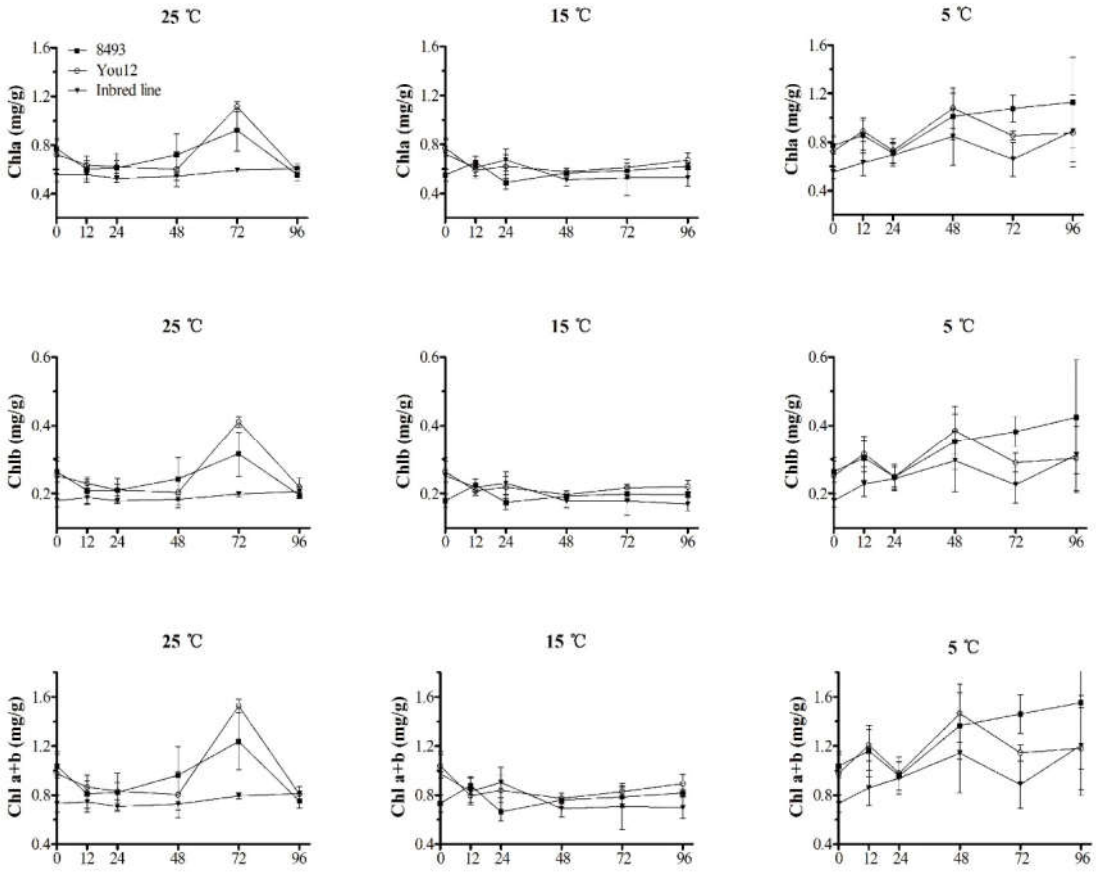

Figure 5.5 The changes in chlorophyll content in leaves of 8493, You12 and Inbred line at 25 °C, 20 °C and 5 °C for 96 h.

*The error bars represent the standard error (±SE) for three replicates.

5.4 Discussion

ChlF is a nondestructive method to evaluate the efficiency of PSII photochemistry, which is already used for high throughput screening and auxiliary diagnosis of both biotic and abiotic stresses (Lootens *et al.* 2011). ChlF emission can detect this harm non-invasively by measuring the response of thylakoid membranes with sustained low-temperature treatments (Baker 2008; Gremer *et al.* 2012). Low temperature stress for ChlF of plants is the result of the combined effect of environmental stress affects and plant response recovery ability (Kalaji *et al.* 2016a). Therefore, ChlF parameters could indicate the degree

of damage to the PSII reaction center and inhibition of photosynthetic activity under low temperature (Mishra *et al.* 2015). In a maize low temperature study, the most obvious response of maize seedlings under low temperature conditions is the reduction of photosynthesis. Parts of reactions ranging from the photosynthetic light reaction to accumulation are regarded being affected by chilling stress. In addition, the reduction of photosynthetic capacity upon exposure to low temperature might be caused by a decrease in the activity of certain enzymes of the C4 cycle and/or of the Calvin cycle (Riva-Roveda *et al.* 2016).

All the response indexes were used to perform an ANOVA analysis of the effects of differential temperatures at 96 h treatments on the 3 lines. The results showed that there were significant differences for F_v/F_m, F_v'/F_m', ΦPSII, qP, NPQ, Chl a/b/a+b and NPQ light intensity at 5 ºC stress, which indicated that there are obvious differences when *Zea mays* ssp. *mexicana* L. is exposed to 5 ºC chilling conditions. However, there were no obvious differences in the 15 ºC group compared to the CK after 96 h treatments (Figure 5.6).

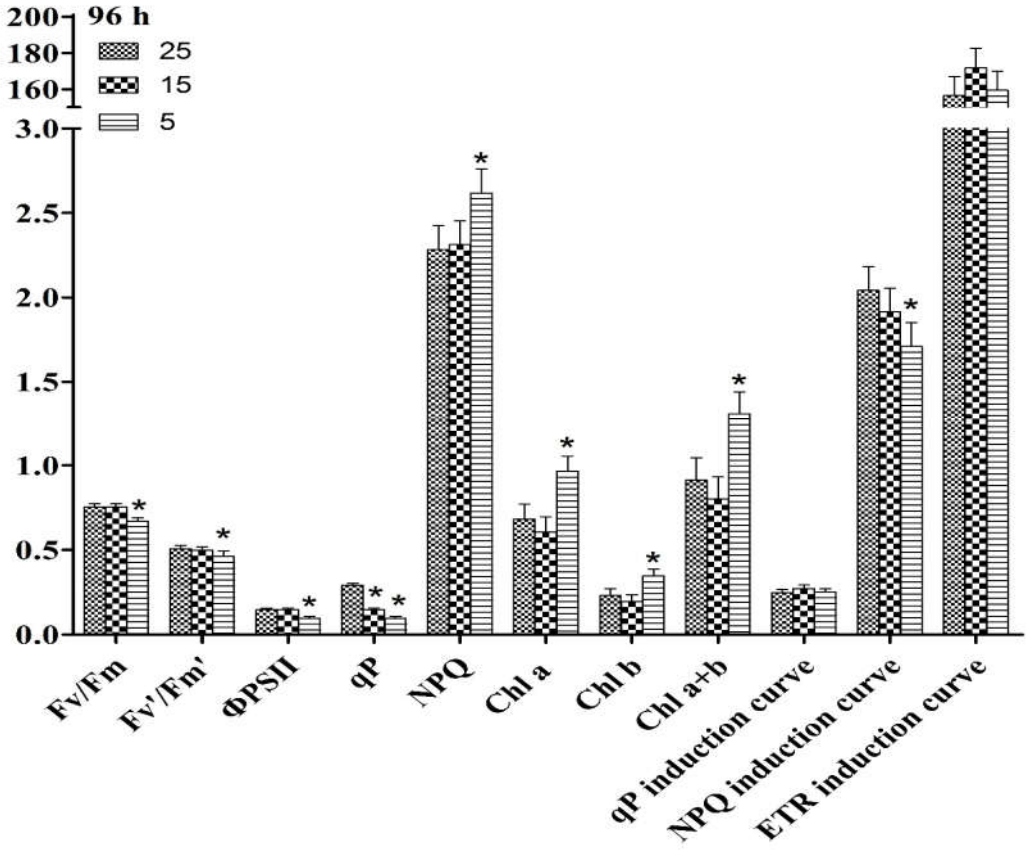

Figure 5.6 The effect of temperature of *Zea mays* ssp. *mexicana* L. under differential chilling stress to 96 h seedlings.

*The mean ± SE followed by different asterisk showed that significant differences ($P \leq 0.05$) used *, according to one-way ANOVA. Each value represents the mean of differential varieties and its standard errors (±SE), LSD 5%.

Under low temperature stress, the decrease of efficiency of PSII leads to the value of F_v/F_m reducing, which represents the maximal quantum efficiency of PSII of dark adapted plants. F_v/F_m and low temperature damage have a linear relationship, including both decreased speed and amplitude. If the degree of damage shows an increase, the value of F_v/F_m will reduce. That reduction is because low temperature harms the photochemical reaction of PSII. It has been identified that F_v/F_m and low temperature damage have a linear relationship. In this study, the ratio of F_v/F_m decreased sharply in the

non-acclimated leaves of the 3 varieties. The results indicated 15 ºC and 5 ºC have differential effects. 15 ºC stress showed no apparent effects. Under 5 ºC stress at 96 h, "8483", "You12" and "Inbred line" were reduced by 11.14 %, 13.65 % and 8.6 % compared to their controls, respectively (Figure 5.7).

According to ANOVA results, all 3 varieties had significant changes under 5 ºC stress. In addition, the results indicated the activity of PSII reaction center declined, and all plants were shown to reach a damaged status, because 0.75-0.85 is a reasonable range for higher plants (Kalaji *et al.* 2016b). The lowest point of "You12" was at 48 h treatment, but it still had a strong recovery ability. Under normal conditions (F_v/F_m undamaged and full dark-adaption), the parameter of PSII has slight changes (Baker & Oxborough 2004). All 3 varieties had the same trend. However, low temperature lead to PSII significantly declining, especially with the 5 ºC stress, with "Inbred line" less affected than the others.

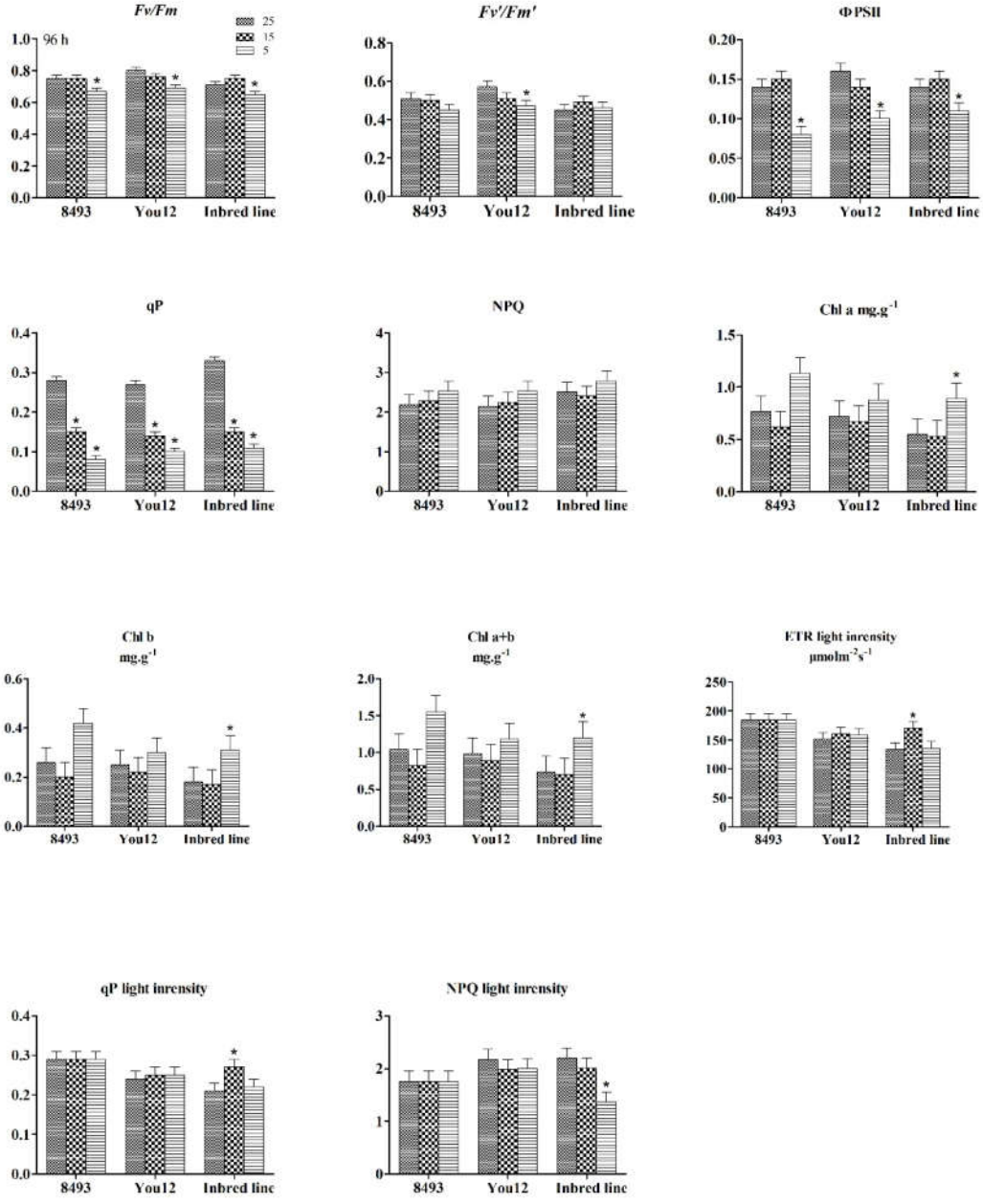

Figure 5.7 The effect of chilling stress (15 ºC and 5 ºC) on the index of Chlorophyll Fluorescence and the content of Chlorophyll in leaf of 3 varieties of *Zea mays* ssp. *mexicana* L.

*Each value represents the mean of three replicates (n=3) and its standard errors (±SE), LSD 5%.

For the light induction parameters, such as ΦPSII and qP the results showed similar declines with time under low temperature stress. 5 ºC low temperature conditions lead to all varieties showing significantly reducing trends, which confirmed that the chilling temperatures enhanced photoinhibition of PSII by increasing the closure of PSII reaction centers, reducing photochemical efficiency relative to the PSII open centers in the light adapted state and PSII maximum efficiency within light-adapted materials. In addition, the decrease in F_v'/F_m' can be attributed to an increasing level of energy dissipation and a reducing utilization of the absorbed energy in the photochemical processes (Turan & Ekmekçi 2011). In the present study, the progressively increasing NPQ with decreasing temperatures are according to the traits of photochemical processes. The 3 varieties all showed no obvious difference of NPQ, which indicated the light protecting abilities were similar.

In this study of 3 varieties of *Zea mexicana* the values of ETR were all reduced with the time course of low temperature stress, though only "Inbred line" at 15 ºC was significantly lower that their un-treated status. That may indicate that the PS II reaction center had suffered some injury, which lead to inhibition of the transfer processes Q_A, Q_B and PQ in the reaction center thereby reducing ETR (Meng *et al.* 2016). Therefore, we suggested that the photosynthetic machinery was less damaged for "Inbred line". In addition, "Inbred line" had relatively higher ETR than the other lines, which revealed that there was less damage to the PSII electron transport under chilling stress.

The photochemical (PSII efficiency factor) and the nonphotochemical processes (NPQ) were significantly affected by the light applied. With the increase in the ML, the NPQ of all 3 varieties showed an increase. Similar phenomenon have been found in *Cichorium intubus* L., *Silene dioica* and grapevines (Baker 1991; Devacht *et al.* 2011; Hendrickson *et al.* 2003). At low temperatures, the maximum NPQ value remained low which indicated that photoprotection was not necessary or that the D1 protein of the PSII was damaged or that other processes may have attended to the energy dissipation at low

measured temperatures (Gombos, Wada & Murata 1994). The minimum PSII efficiency factor qP decreased with decreasing MT and increasing ML. Thus the decrease of qP may have limited dark reactions, as NADPH accumulates and thus limits the electron transport. This phenomenon was identified by Lichtenthaler and Burkhart (Lichtenthaler & Burkart 1999). In addition, the results showed that low temperature caused the reduction of ETR_{max}, which indicated that low temperature affected light energy utilization ability and 5 ºC showed more serious effects than 15 ºC stress. Under low temperature conditions, plants have to release excess energy in order to avoid inactivity or damage of PSII.

In other studies, the content of Chl a/b, a+b were shown to reduce gradually over time with low temperature stress (Wenhai & Jingquan 2001). The increasing content we found in this study suggests low temperatures affected the photosystem of *Zea mexicana* and was associated with an increase in protective mechanisms. Our results showed that "Inbred line" has obvious differences under 5 ºC stress, but other varieties were not as obviously affected. These results suggested that the treatment times with low temperature were not long enough. In addition, "Inbred line" reduced chlorophyll contents between 48 h to 96 h. Compared to maize research results (Leipner 2009), the chl a/b of maize were decreased significantly at 5 ºC after 96 h of stress, but different results showed in our research. We suggest that the low temperature tolerance is better than maize, which lead to the chl a/b still showing increasing values compared to the same treatments in maize. Our results suggest that the protection processes of the low temperature responses for *Zea mexicana* last longer than with maize for the same time course.

The protocol employed in this work; the use of plants in an early stage and a relatively modest time course, makes it possible to achieve rapid and credible results and to handle a large number of genotypes as would be needed for experiments required in breeding activities. Compared with the chlorophyll test, the *Fv/Fm* method is not destructive and requires less work (Bolhar-Nordenkampf *et al.* 1989; Schreiber, Bilger & Neubauer 1995). For these reasons it is particularly suitable to assess the relative ranking of a large number

of genotypes. Despite some limitations, low temperature evaluation experiments represent an available tool for crop genetics breeding and for the evaluation of biodiversity. In this context, according to our results, the response of *Zea mexicana* is significantly dependent on the form of application of the cold conditions. The photosynthesis of all lines was strongly affected by low temperature. The *Fv/Fm* and other photosystem parameters analyses are an effective test because they allow monitoring of the capacity to maintain a functional PSII during low temperature treatments, which may be associated with a crucial trait for the acquisition of chilling tolerance.

5.5 Conclusion

The main objective of this experiment was to investigate the use of ChlF methods to study the differential low temperature tolerance of different *Zea mexicana* varieties (8493, You12 and Inbred line) at the seedling stage. We used Chl *a* fluorescence imaging to evaluate the tolerance of *Zea mexicana* plants to the particular low temperature stress conditions investigated. In this article, the parameters related to the light induction kinetics of the ChlF process were analyzed and discussed in detail. Our results showed that "Inbred line" variety was more sensitive than the other tested varieties. Consequently, a better understanding of the chilling mechanisms will provide valuable information for designing better selection strategies, and speeding up breeding in the future. In addition, we will use our cold stress RNA-Seq data combined with our cloned cold related key genes to carry out further gene functional studies, and use transgenic technology for improving cold tolerance of *Zea mexicana* cultivars and other *Zea mays* crops to increase the chilling stress tolerance capacity of chilling-sensitive plants.

Chapter 6 Functional characterization a novel SUMO E3 ligase ZmmSIZ2 from *Zea mays* ssp. *mexicana* L.

Abstract

The annual *Zea mays* ssp. *mexicana* L., a member of teosinte, has strong growth and regeneration ability, high tiller numbers, high protein and lysine content as well as resistance to many fungal diseases. It is a close wild relative of maize thus it can be effectively used in maize improvement. As important forage resources, cold tolerance is an important trait for teosinte breeding, so study molecular mechanism of key pathway genes have greatly potential benefits. In this study, we showed that the *Zea mays* ssp. *mexicana* L. gene *ZmmSIZ2*, a SUMO E3 ligase, was isolated from a cDNA library of RNA-Seq from cold-treated seedlings tissues of *Zea mays* ssp. *mexicana* L. It is an important regulator of ICE1 activity and regulates CBF3/DREB1A and downstream target gene expression. The deduced protein for *ZmmSIZ2* contains a highly conserved MIZ/SP-RING zinc finger domain for SUMO E3 ligase activity and binding to SUMO E2, a helix-extended loop-helix SAP (scaffold attachment factors SAF-A/B, Acinus, PIAS) domain for DNA binding and PHD domain. The ZmmSIZ2 protein shows sumoylation when expressed in an *Escherichia coli* reconstitution system.

Keywords *ZmmSIZ2*, SUMO E3 ligase, *Zea mays* ssp. *mexicana* L.

6.1 Introduction

Zea mays ssp. *mexicana* L. a member of teosintes, is a wild grass native to high altitude in northern and central Mexico (about 1,600~2,700 m) with a large spikeleted trait and is a close wild relative of cultivated maize (*Zea mays* ssp. *mays* L.), with which it has normal chromosome pairing in meiosis and general fertility (Fukunaga *et al.* 2005). It is an important genetic material for the improvement of agronomic characteristics of maize and teosinte genetics, genome evolution and breeding (Cohen & Galinat 1984; Reeves 1950; Takahashi *et al.* 1999; Wang *et al.* 2008). The annual *Zea mays* ssp. *mexicana* L. has stronger growth ability and regeneration ability, more tillers, higher protein content in the kernel and more dominant resistance to many fungal diseases than cultivated maize. It can be used to feed livestock directly or be reserved as silage and hay (Fang *et al.* 2012; Fukunaga *et al.* 2005; Hufford *et al.* 2012; Mangelsdorf 1961; Wllkes 1977). However, most lines of *Zea mays* ssp. *mexicana* L., originated from subtropical areas of South American are sensitive to low temperature stress (Hincha & Zuther 2014). Thus, the understanding of the molecular mechanisms of key cold resistance genes of *Zea mays* ssp. *mexicana* L. may significantly increase new strategies for improving the yield of low temperature sensitive agronomic plants and expanding the geographic districts of production.

In eukaryotes, post-translational processes modify diversify of the proteome by glycosylation, phosphorylation, acetylation, methylation, or ubiquitination by Ub (ubiquitin) and Ubls (ubiquitin-like proteins) activity to mediate complex hierarchical regulatory processes that are crucial to eukaryotic cell function. These help improve adaptability and resistance development to environment change (Miura & Hasegawa 2010). Similar to ubiquitination, Sumoylation, is known to facilitate reversible conjugation of a SUMO (small ubiquitin-related modifier) polypeptide to protein substrates. SUMO conjugation to protein substrates (sumoylation) is an inevitable protein post-translational modification which begins with the activation of the SUMO C-terminal by an E1 activating

enzyme, a subsequent transfer to an SUMO E2 conjugating enzyme, and then with the help of an E3 ligase, SUMO is finally conjugated to a substrate protein. Sumoylated proteins can be removed from conjugates by SUMO proteases that are responsible for SUMO recycling (Zhang *et al.* 2013b). It represses or facilitates transcription factor activity by either SUMO E3 ligase dependent or independent mechanisms under environmental stimuli in animals, plant and yeasts (Hay 2005; Miura *et al.* 2007). Similar to ubiquitin, SUMO conjugation influences transcription factor function by activation, repression, or protein stabilization processes. Sumoylation/desumoylation of substrates are used as an important regulatory process in plant development processes, such as innate immunity, chromosome segregation and cell division, DNA repair, nucleo-cytoplasm trafficking, subnuclear targeting transcriptional regulation, ubiquitin-mediated protein degradation by proteasomes, phosphate deficiency, genotoxic stress and responses to abiotic stresses, including drought, ABA signaling, and low temperature response (Chiou & Lin 2011).

In plants, homology studies have identified SUMO machinery members and most of the *Arabidopsis* orthologues have been biochemically validated. *Arabidopsis* SIZ1-Mediated sumoylation has been identified as a SUMO E3 ligase that regulates cold acclimation by controlling ICE1 activity, CBF/DREB1 expression (particularly CBF3/DREB1A), and downstream target gene function (Miura *et al.* 2007; Miura *et al.* 2005). Moreover, *Arabidopsis* SIZ1 controls cell growth and plant development through salicylic acid (SA). The *siz1* mutant plant showed dwarfism traits of reduced leaf size, cell size and cell number, which is due to negative regulation of SA-mediated inhibition of cell division and elongation (Miura *et al.* 2010; Niazi *et al.* 2015b). Although many genetic studies have established a role for SUMOylation in defense, abiotic stress responses, hormone signalling and flowering control, and SUMOylation as being essential during seed development, much less is known about the molecular mechanisms through which SUMO regulates those biological processes.

In this study, we identified a SUMO E3 ligase *ZmmSIZ2*, which contains a highly

conserved MIZ/SP-RING zinc finger domain for SUMO E3 ligase activity and binding to SUMO E2, a helix-extended loop-helix SAP (scaffold attachment factors SAF-A/B, Acinus, PIAS) domain for DNA binding and plant specific PHD (plant homeodomain) domain, which is a C_4HC_3-type RING figuer relates to chromatin remodeling and ubiquitin E3 ligase activity, from *Zea mays* ssp. *mexicana* L. *ZmmSIZ2* sumoylation modification process. These results have provided some additional biochemical evidence for ZmmSIZ2 functioning and the next step is to obtain overexpression lines for understanding the molecular basis of cold tolerance mechanisms. These data can offer better reference information for understanding teosinte and other crops.

6.2 Materials and methods

6.2.1 Plant materials, growth conditions and RNA isolation

The seeds of *Zea mays* ssp. *mexicana* L. variety "8493" were obtained from Guangdong Provincial Key Laboratory of Biotechnology for Plant development of the School of Life Science of South China Normal University (SCNU, Guangzhou, Guangdong province, China). After being sterilized, plump seeds were planted in plastic boxes (54 cm length, 28 cm width and 7 cm height) with soil substrates (Jiffy, Netherlands). These germination methods are according to the CIMMYT operation manual (Taba *et al.* 2004b) . The boxes were placed in a climate control box (RXZ 500-C, JIANGNAN Instrument) (25 °C under a 10-h light/14-h dark photoperiod, humidity of 60%). Twenty-one days old seedlings materials were treated at 4 °C for the cold stress and then frozen in liquid nitrogen for total RNA isolation. Total RNA of mixed samples was extracted by TRIzol® reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. RNA was purified with RNase-free DNase I and used for reverse transcription by PrimeScript™ RT reagent Kit (Takara, Japan). *Arabidopsis thaliana* (WT: Columbia 0 and Mutant: ICE1-2, Salk_003155) were grown for genetic transformation (22 °C under a 16-h light/8-h dark photoperiod, humidity of 40%).

6.2.2 Isolation of the full-length cDNA of *ZmmICE-like* and Bioinformatics analysis

To determine the expression profiles of *ZmmSIZ2* of *Zea mays* ssp. *mexicana* L. in *Arabidopsis* plants, total RNAs and cDNA were prepared as described above. Based on our unigenes *de novo* sequence by RNA-Seq and nucleotide sequences BLAST results, the CDS sequence was confirmed. PCR was performed using primer pairs specific for full-length *ZmmSIZ2* gene, with forward primer 5'-AAGCTTATGGACAACTCGAAGGAGGTAAAGC-3'; and reverse primer 5'-TCTAGATTACATTGCATTGTGGAGACCGG-3', (The added restriction sites *Hind III* and *Xbar I* were underlined, respectively). The PCR program was as follows: 98 °C, 3 min; 30 cycles, 98 °C, 30 s; 60 °C, 30 s; 68 °C, 60 s; 68 °C, 10 min. The completed *ZmmSIZ2* was cloned into the *pEASY*®-Blunt Simple Cloning Vector (TransGen Biotech, Guang Zhou) for sequencing. Then the PCR products were cloned into pCAMBIA 1300-221 vector and sequenced. The online program Computer pI/Mw (http://web.expasy.org/compute_pi/) was used to predict the theoretical pI (isoelectric point) and MW (molecular weight) of the *ZmmSIZ2* protein. Phylogenetic tree was based on the deduced amino acid sequences of *SIZ2* from a range of plant species by MEGA 5.1 with 500 bootstrap tests by Neighbor-joining tree. ClustalX 1.8 and DNAman 8.0 alignment were employed for deduced *ZmmSIZ2* amino acid sequence homology alignment. Predicted molecular model building of *ZmmSIZ2* was carried out using phyre2 online server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

6.2.3 Western blot protocol and sumoylation assay *in vivo*

In order to analyse *ZmmSIZ2* sumoylation processes in an *in vitro* prokaryotic expression system, full-length *ZmmSIZ2* was PCR amplified with the primers 5'-G/AATTCATGGCGTCGCCGGACGA-3' (*Ecor1*) and 5'-GTCGACTTAATCAGAATCTGAGGAAATCTCTAGTAT-3' (*Sal1*). We cloned *ZmmSIZ2* into the prokaryotic expression vector PEGX-4T-1 (Novagen, Germany) for as a fusion vector with His-Tag and expressed in bacteria carrying

pET28-SAE1a-His-AtSAE2 (E1) and *pACYCDuet-1-SCE1-SUMO1 (GG)* (E2 and SUMO). The transformed cells were cultured in LB medium with required antibiotics to OD600 of 0.5 and induced by 0.2 mM IPTG. After incubation for 12 hours at 25°C, cells were harvested and used for immunoblotting. The resultant proteins were separated with SDS-PAGE and blotted onto a PVDF membrane (Immobilon, USA). The gel blot was probed with anti-GST (TransGen, China) antibodies and visualized by chemi-luminescence using the ECL plus kit (Cwbio, China) according to the manufacturer's instructions.

6.3 Results

6.3.1 Isolation and characterization of *ZmmSIZ2* gene

We identified a putative *Zea mays* ssp. *mexicana* L. *SIZ2* gene based on the conserved domains of *SIZ2* homology in several plant species. The full length cDNA sequence of the *ZmmSIZ2* gene was amplified. The total base number of the complete cDNA of *ZmmSIZ2* is 2,481 bp and the cDNA encodes a predicted protein of 827-amino acid polypeptides. The predicted molecular weight of *ZmmSIZ2* is 90.324 kDa and a pI of 5.18. The complete *ZmmSIZ2* protein has 99% homology with the Maize putative E3 SUMO-protein ligase SIZ2-like isoform X2, which suggested that *ZmmSIZ2* was an ortholog of SIZ2.

After BLAST search with different plants sequences, the highest query coverage sequences were extracted for sequences and homology analysis. The result indicated that *ZmmSIZ2* contains the same conserved MIZ/SP-RING zinc finger domain, helix-extended loop-helix SAP (scaffold attachment factors SAF-A/B, Acinus, PIAS) domain and plant specific PHD (plant homeodomain) domain as other SIZ1/SIZ2 proteins. To investigate the evolutionary relationship among the SIZ1/2-like proteins, a phylogenetic tree was constructed in terms of their complete CDS sequences. ZmmSIZ2 has high homology with maize, rice, wheat and *Brachypodium distachyon*. These results indicated that ZmmSIZ2 has typical features of SIZ1/2-like proteins and was closely related to some homologues in monocots (Figure 6.1).

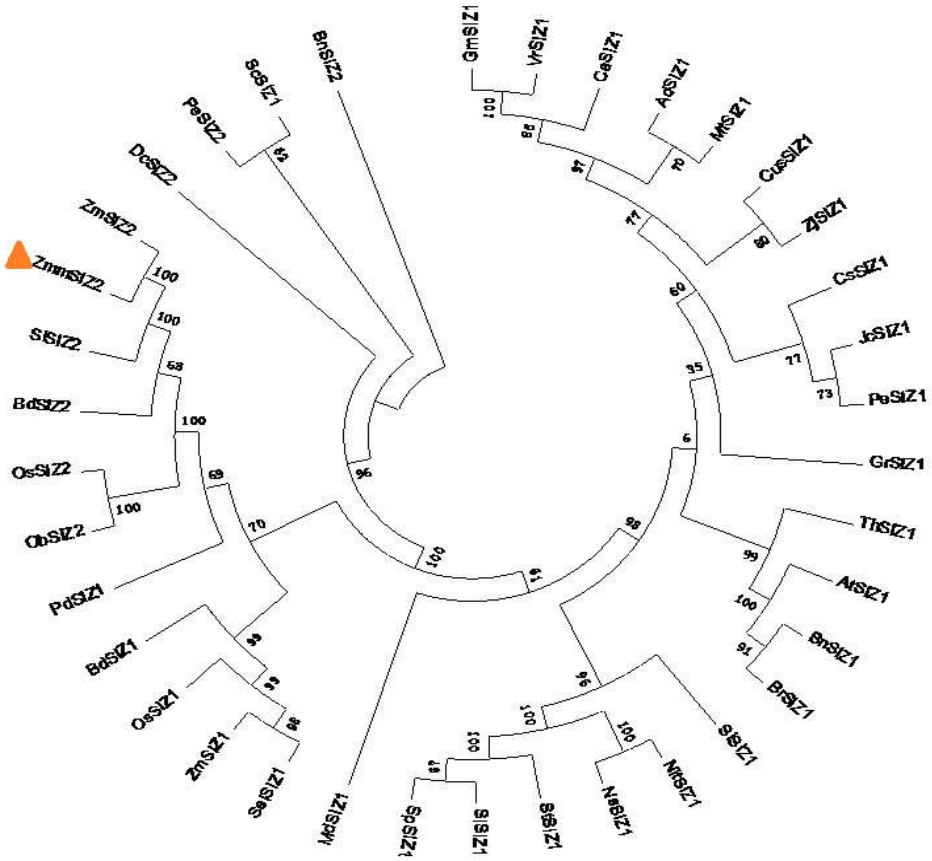

Figure 6.1 (A) Phylogenetic tree based on the deduced amino acid sequences of SIZ1/2 from a range of plant species.

*The origin and GenBank accession numbers of the compared sequences are as follows: AtSIZ1 [*Arabidopsis thaliana*] (NM_125434.3); SlSIZ1 [*Solanum lycopersicum*] (NM_001328641.1); OsSIZ1 [*Oryza sativa*] (XM_015784156.1); MtSIZ1 [*Medicago truncatula*] (XM_003606406.2); GmSIZ1 [*Glycine max*] (XM_006594929.2); VrSIZ1 [*Vigna radiata*] (XM_014642186.1); GrSIZ1 [*Gossypium raimondii*] (XM_012607261.1); SiSIZ1 [*Sesamum indicum*] (XM_011091036.1); PeSIZ1 [*Populus euphratica*] (XM_011042568.1); NsSIZ1 [*Nicotiana sylvestris*] (XM_009789426.1); NtSIZ1 [*Nicotiana tomentosiformis*] (XM_009619452.1); ZmSIZ1 [*Zea mays*] (XM_008658310.1)**;** CsSIZ1 [*Citrus sinensis*] (XM_006488077.2); StSIZ1 [*Solanum tuberosum*] (XM_015314994.1; SiSIZ2 [*Setaria italica*] (XM_004960295.1); CsSIZ1

[*Cucumis sativus*] (XM_004147219.2)**;** BdSIZ1 [*Brachypodium distachyon*] (XM_010234592.2); AdSIZ1 [*Arachis duranensis*] (XM_016080248.1); ZjSIZ1 [*Ziziphus jujuba*] (XM_016025005.1)**;** SpSIZ1 [*Solanum pennellii*] (XM_015202743.1); BnSIZ1 [*Brassica napus*] (XM_013842032.1); JcSIZ1 [*Jatropha curcas*] (XM_012209470.1); ThSIZ1 [*Tarenaya hassleriana*] (XM_010522046.1)**;** BrSIZ1 [*Brassica rapa*] (XM_009128359.1); PdSIZ1 [*Phoenix dactylifera*] (XM_008785503.1); MdSIZ1 [*Malus x domestica*] (XM_008378068.2); CaSIZ1 [*Cicer arietinum*] (XM_004487079.2); ZmSIZ2 [*Zea mays*](XP_008675556.1); OsSIZ2 [*Oryza sativa*] (XM_015776689.1); BdSIZ2 [*Brachypodium distachyon*] (XM_003577117.2); DcSIZ2 [*Daucus carota*] (XM_017366318.1); BnSIZ2 [*Brassica napus*] (XM_013856275.1); PeSIZ2 [*Populus euphratica*] (XM_011024311.1); ZmSIZ2 [*Zea mays*] (XM_008677334.1); ObSIZ2 [*Oryza brachyantha*] (XM_015843099.1); Sisiz2 [*Setaria italica*] (XM_004977584.2) and ZmmSIZ2, was produced by MEGA 5.1 with 500 bootstrap tests by Neighlbor-joining tree.

To investigate the evolutionary relationship among the SIZ1/2-like proteins, a phylogenetic tree was constructed in terms of their complete CDS sequences. *ZmmSIZ2* showed high homology with *Zea mays*, *Setaria italic*, *Brachypodium distachyon*, *Oryza sativa*, *Triticum urartu*, *Aegilops tauschii*, *Phoenix dactylifera*, *Ziziphus jujube*, *Populus euphratica*, *Tarenaya hassleriana*, *Erythranthe guttata*, *Arabidopsis thaliana* and *Vitis vinifera*. These results indicated that Zmmsiz2 has typical features of siz1/2-like proteins and is closely related to some homologues in monocots (Figure 6.2). The predicted three dimensional structures of the ZmmSIZ2 were distinct, and were more similar to the structures of fold library id: c3i2dA (Figure 6.3).

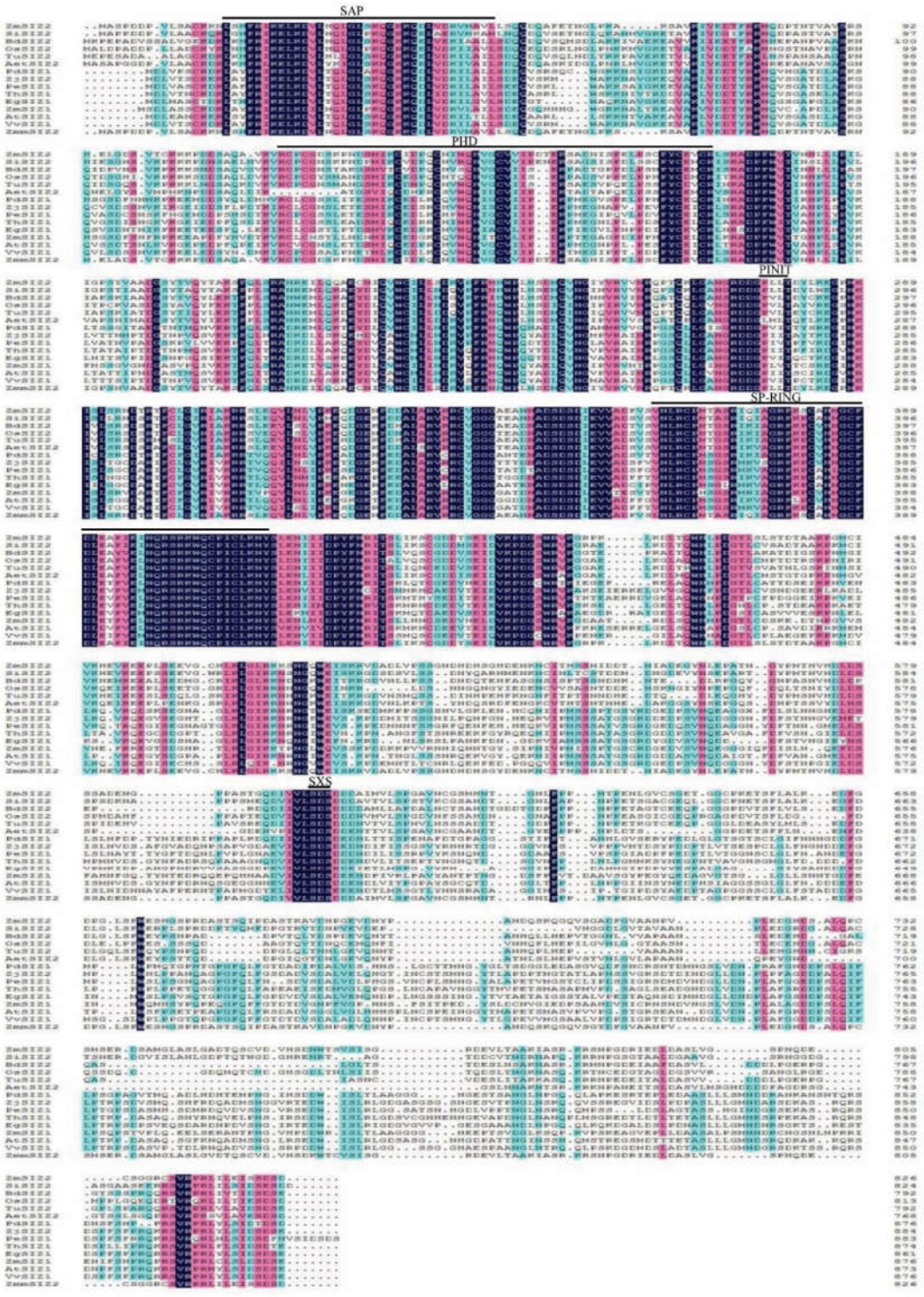

Figure 6.2 Comparison of ZmmSIZ2 amino acid sequences with homologous proteins. *ClustalX 1.8 and DNAman 8.0 alignment were employed for deduced sequence homology alignment. The Sequences and accession numbers are shown for the following:

ZmSIZ2 [*Zea mays*] (XP_008675556.1); SiSIZ2 [*Setaria italica*] (XP_004977641.1); BdSIZ2 [*Brachypodium distachyon*] (XP_003577165.1); OsSIZ2 [*Oryza sativa*] (XP_015632175.1); TuSIZ2 [*Triticum urartu*] (EMS63883.1); AetSIZ2 [*Aegilops tauschii*] (EMT28925.1); PdSIZ1 [*Phoenix dactylifera*] (XP_008789064.1); ZjSIZ1 [*Ziziphus jujuba*] (XP_015880489.1); PeSIZ1 [*Populus euphratica*] (XP_011040866.1); ThSIZ1 [*Tarenaya hassleriana*] (XP_010535588.1); EgSIZ1 [*Erythranthe guttata*] (XP_012827801.1); ZmSIZ1 [*Zea mays*] (XP_008656531.1); AtSIZ1[*Arabidopsis thaliana*] (NM_125434.3); VvSIZ1 [*Vitis vinifera*] (XP_010651133.1) and ZmmSIZ2. The domains include: the SAP (Scaffold attachment factor A/B/acinus/PIAS); the PHD (Plant homeodomain); the PINIT (Pro-Ile-Asn-Ile-Thr); the SP-RING (SIZ/PIAS-RING); and the SXS (Ser-X-Ser) domain. Residues in black and red regions indicate identical and similar residues, respectively, between isoforms.

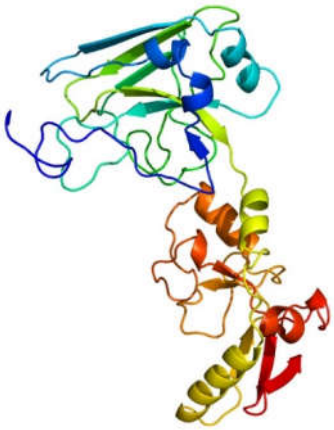

Figure 6.3 The predicted tertiary structures ZmmSIZ2 protein evaluated by the phyre2 online server.

6.3.2 Sumoylation assay of ZmmSIZ2

In order to detect the sumoylation of ZmmSIZ2, we performed sumoylation assays in an *E.coli* strain expressing *ZmmSIZ2* together with AtSAE1a-SAE2, AtSCE1a and AtSUMO1

(Liu *et al.* 2015; Zhang, Qi & Yang 2010). The results showed that SUMO small molecules can covalently bind to the target gene when Sumo E1 and Sumo E2 exist. Thus, *ZmmSIZ2* has sumoylation specific binding sites, which is in accordance with its *SIZ1/2*-like features and bioinformatical predicted outcomes (Figure 6.4, Figure 4.3).

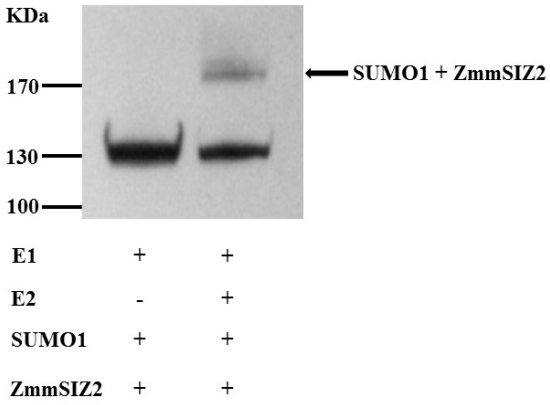

Figure 6.4 ZmmSIZ2 sumoylation *in vitro* assay result.

*PEGX-4T-1-GST-ZmmSIZ2 were expressed in *E. coli* and then tested for SUMOylation activity. Immunoblots generated from these samples were probed with anti-GST antibodies.

6.4 Discussion

SUMOylation is a post-translational regulatory process in eukaryotes. Small ubiquitin-related modifer (Matsumoto *et al.*), a key regulator of biological processes, is covalently conjugated to a lysine residue in a substrate protein via a reversible posttranslational modification process that is facilitated by E3 SUMO ligases. In plants, SUMOylation has mostly been studied in model plant *Arabidopsis*, such as ABA responses, flowering time, phosphate starvation responses, salicylic acid (SA)-dependent defense responses, stress and defence responses basal thermo-tolerance and removal of heavy metal (Lee *et al.* 2007; Park, Song & Seo 2011). In the study of monocotyledons, only OsSIZ1/2 and DnSIZ1 were described from *Oryza sativa* and *Dendrobium*, respectively (Liu *et al.* 2015; Park *et al.* 2010). It has been suggested that they have homologous functions with *Arabidopsis* AtSIZ1

in the SUMO conjugation.

In this current study, a *Zea mays* ssp. *mexicana* L. SIZ2 gene, *ZmmSIZ2*, was identified based on homology to SIZ genes from *Zea mays*, *Arabidopsis thaliana*, *Vitis vinifera, Populus euphratica, et al* (Figure 6.2). The SAP, SP-RING, SXS, PINIT and PHD domains of ZmmSIZ2 have a high degree of sequence conservation with those of ZmSIZ2, while the domain is most similar to that of the ZmSIZ1/2, which has the canonical SP-RING domain that is necessary for the SUMO E3 ligase activity of SIZ/PIAS proteins. We speculate that ZmmSIZ2 encodes a SPRING protein with SUMO E3 ligase activity.

Little is known about the function of ZmmSIZ2 and the biological significance and SUMOylation mechanism in *Zea mays* ssp. *mexicana* L. In this study, we constructed a SUMOylation reactions system in *E.coli* and identified that ZmmSIZ2 can conjunct with SUMO1 in an *in vitro* assay as a functional SUMO E3 ligase. We suggested that ZmmSIZ2 plays an important role in the regulation of *Zea mays* ssp. *mexicana* L. stress responses, flowering and development.

6.5 Conclusion

We identified a SUMO E3 ligase that encodes SAP, SP-RING and PHD domains named as *ZmmSIZ2*, from *Zea mays* ssp. *mexicana* L. *ZmmSIZ2* participates in a sumoylation modification process. It can also offer better reference information for future functional study.

Chapter 7 General discussion

7.1 Background

The importance of forage (particularly greenfeed, *Zea mays* ssp. *mexicana* L. to modern agricultural production and livestock breeding has been described in Chapter 1. In order to meet the needs of agriculture and consumers the creation, selection and fixation of the requirements for plant phenotypes using plant breeding for improving plant materials is the primary task (Moose & Mumm 2008). Traditional breeding projects are focused on increasing productivity and quality and disease resistance. With the development of molecular biology it has been shown that traditional breeding methods through phenotypic selection cannot alone satisfy all the requirements of breeding. Thus, modern cultivars have limited tolerance of abiotic stresses, which greatly affect the huge productivity requirements in the world and in China. In order to resolve these problem in the shortest possible time, the introgression of abiotic stress tolerance into modern cultivars is essential work for the future (Dunwell 2014). Until now, phenotype selection, marker assisted selection (MAS), and genetic engineering have been the main methods used to produce low temperature tolerant cereals. In addition, exploring available germplasm resources in wild relative species is becoming a high value activity. In plant, crop and forage studies, it has been shown that wild relatives have a rich genetic diversity with characteristics of wildness, aggressiveness and resistance. Therefore they are usually used as valuable original materials for genetics, breeding and natural gene banks for locating and adding new diversity to cultivated cultivars, which resolves the problem of limited genes available in traditional gene pooling for traditional genetic breeding (Inoue, Fujimori & Cai 2007).

Cold tolerance is an important ability of plants to defend against temperature change and is an irreplaceable trait in worldwide production of many crops. In different districts, plants need to develop lots of strategies to adjust to cold stress for living by promoting a series of complex processes of physiological rearrangement (Mishra, Heyer & Mishra 2014). The low temperature injury of plants includes chilling injury (>0 °C) and freezing

injury (<0 °C) (Verslues *et al.* 2006). These two stresses can greatly reduce plant growth and development (Ensminger, Busch & Huner 2006). Low temperature can lead to formation of ice crystals in the cell, which affects the structure of cell membranes and makes cells lose function. Cells can reduce the damage of chilling injury or freezing injury by improving sugar content, increasing antioxidants, taking mechanical action and inducing molecular chaperone chemicals (Leyva *et al.* 1995). Meanwhile, metabolic processes of cell membrane can also be changed by low temperature, such as photosynthesis and respiration (Ryan 1991). The reaction center and light-harvesting antennas of the basic photosynthesis membrane system lie on chloroplast thylakoid phospholipid bilayer-membranes. These attend the process of transforming light energy into biochemical usable chemical potential energy (ATP) and redox potential energy (NADPH) (Ensminger, Busch & Huner 2006). Under low temperature stress, the structural stability of the lipid bilayeris reduced. Especially the photosynthesis PSII reaction center can be harmed under this stress (Huner *et al.* 1993).

In addition, low temperature can also change the activity and the reaction speed of enzymes and metabolism. When plants detect a low temperature signal, different kinds of defensive pathway are activated. These include physical structure adaptation, changing the content of the cell membrane and the arrangement of the cell cytoskeleton; osmoregulation leading to osmotic chemical concentration increase, with soluble sugar, betaine, proline, free amino acids, and ions; antioxidant composition and reactions increase and changes of a number of kinases (Kaplan *et al.* 2004; Levitt 1980). According to previous research results, more than 70% of 400 types of metabolite products can be induced by low temperature (Cook *et al.* 2004b). Soluble proteins, soluble sugars and proline are the most important metabolites for osmoregulation. These methods and vitamin E changes also can reduce plant injury under low temperature (Maeda *et al.* 2006).

In order to better understand cold or drought tolerance in plants and use it for tolerant crops breeding, many genes have been isolated from maize which then rely on the results

from model plant *Arabidopsis* and tobacco to ascertain their functions. Such genes includes *ZmMKK4*, *ZmCPK4*, *ZmLEA5C*, *ZmNAC55*, *ZmDBP4*, *ZmDBP3*, *ZmCLC-d*, *ZmMPK4* and *ZmDBF3* (Jiang *et al.* 2013; Kong *et al.* 2011; Liu *et al.* 2014; Mao *et al.* 2016; Wang & Dong 2009; Wang *et al.* 2015b; Zhou *et al.* 2016; Zhou *et al.* 2012), which have all been implicated in improving cold or drought tolerance of target plants significantly. However, no tolerance related genes have been isolated from teosinte except a tb1 (teosinte branched 1) gene, which has been extensively studies (Clark *et al.* 2006; Vann *et al.* 2015). Actually, the creation of pasture germplasm resources with molecular sequencing technology has seldom been reported for forage studies. Combining pasture germplasm resources and new generation genetics technologies (Transcriptome, Gene expression profiles analysis, Functional gene analysis) will offer genetics related reference information for the creation of pasture breeding information and improving breeding efficiency. At the transcriptional level, use of transcriptome sequencing and digital gene expression profile analysis of plant resistance gene expression will benefit researchers through constructing related tolerance gene resources banks, which will provide important reference information. Meanwhile, gene functional research will be expanded from *Arabidopsis* to include *Zea mays* ssp. *mexicana* L. Until now, few molecular biological studies have been done related to this plant. So these researches will improve the level of knowledge and offer molecular evidence for future breeding strategies of *Zea mays* ssp. *mexicana* L.

7.2 Contribution of this study

7.2.1 Overview

Taken as a whole, we studied a close wild relative of *Zea mays*, *Zea mays* ssp. *mexicana* L. across a range of different aspects. Bioinformatics, gene function and germplasm physiological feature studies showed that investigated the cold tolerance of *Zea mays* ssp. *mexicana* L. were carried out. These results will provide information and methods to breeders for speeding up forage and *Zea mays* genetic breeding time.

7.2.2 Summary of outcomes

1. For the first time, a full of cold/drought induced transcriptome of *Zea mays* ssp. *mexicana* L. at the RNA level was created. A large number of DEGs and biological processes transcripts were found. The results showed that cold response is more sensitive than drought response with GO enrichment producting this result, which was similar to previous studies in maize. Some significant functional enrichment analyses identified many common or specific gene sets, biological processes and mechanisms in response to drought and cold stresses were identified that were additional to those found in maize. These included pathways such as the ABA dependent pathway, trehalose synthetic pathway and the ICE1-CBF pathway.All of these were up-regulated by both stresses. GA associated genes have been shown to differentially regulate the responses to cold in the close subspecies in *Zea mays*. Based on these findings it was suggested that improving cold/drought tolerance studies will need increasing numbers of experiments under different conditions for finding more clues to understand genetic response to stress in future studies.

2. According to RNA-Seq data, the DEGs and CDS dataset were used as fragments of information for target genes assembly. When compared with the NCBI database, we used the above results to assemble *ZmmICE1* and *ZmmSIZ2* successfully. After bioinformatics analysis, the structural features of these two genes were found to lie between monocotyledon and dicotyledon examples and the sumyolation modifications were detected by our in vivo detecting system. Experiments identified that SUMO small molecules can covalently bind to both of these target genes when Sumo E1 and Sumo E2 exist. In addition, the function of ZmmICE1 was identified in transgenic *Arabidopsis thaliana*. The cold tolerance was improved significantly and substantially in transgenic lines. These results showed that *ICE* family genes were functioning in low temperature tolerance in *Zea mays* ssp. *mexicana* L. and the results substantially increase our understanding of the molecular basis of cold tolerance mechanisms. These data can also offer better reference information for understanding teosinte and other crop cold tolerance

mechanisms.

3. We used ChlF technology detect seedling photosynthesis responses of varieties of *Zea mays* ssp. *mexicana* L. with different induced chilling tolerances. The results showed that the chilling sensitive of "Inbred lines" was better that of the commercial varieties "8493" and "You12", which provides available references for future teosinite breeding.

7.3 Suggested further studies

Therefore, the collecting, analysing and studying of wild forage germplasm can enrich and accumulate forage germplasm resources for the future development and utilization. In this thesis, we combing bioinformatics, molecular biology and physiological research to deeply study the low temperature tolerance of *Zea mays* ssp. *mexicana* L. We have output some important benefits for understanding cold/drought functional mechanism of future of *Zea* breeding:

7.3.1 The value of RNA-seq data

The RNA-seq analyse provide highly valuable data for *Zea* study. The data had been uploaded into the NCBI GEO database (accession number: GSE76939) and now exists as a public data set for any researchers who have interests in studies of the genus *Zea* or wider studies of gene functionality study and abiotic stress tolerance. The data including cold/drought induced genes information and the functional cluster information, can be used to guild gene assembly and cloning, functional studies and multi-gene co-transformations in homologous and heterologous backgrounds.

7.3.2 Identification of cloned genes and sumoylation detection

For cloned studies in this research, we cloned the *ZmmICE1* and *ZmmSIZ2* genes for use with functional and freezing studies. In general, most ICE1 homologous gene predicted sumoylation sites were predicted by bioinformatics sequences analysis, however, we used our *in vivo* sumoylation detecting system which identified sumo function sites, which can

be used as references in homologous genes for enriching research contents. In addition, we identified that there are no interactions between *ZmmICE1* and *ZmmSIZ2* in the process of protein post-translational modification in *Zea mays* ssp. *mexicana* L.

7.3.3 The utilization of future varieties

We compared to the chilling tolerance of differential seedlings varieties by photosystem response. The varieties of “8493” and “Inbred line” were regarded as a potential available variety with cold tolerance for use in future forage breeding. Based on this chilling tolerance trait, breeders can use these varieties as a good parent for further improving yield performance using shortages by transgenic technology and traditional breeding processes.

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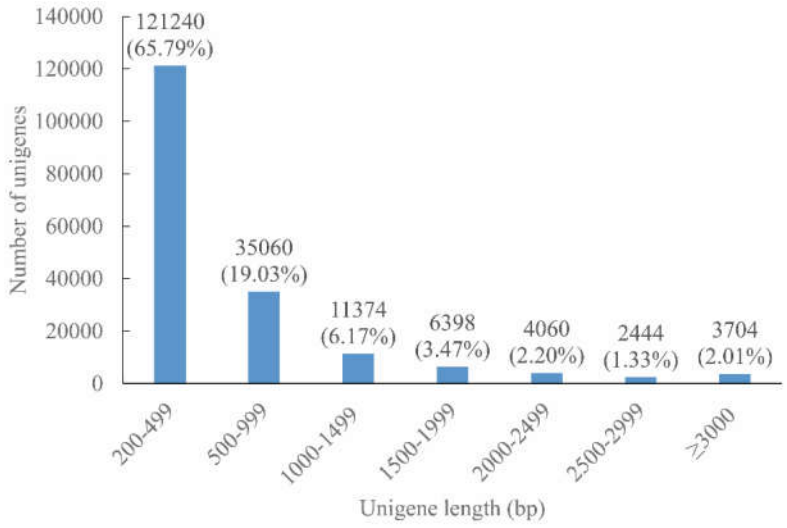
Supplemental files

Supplemental file 1 Distribution of the length of transcripts and unigenes.

Distribution of length

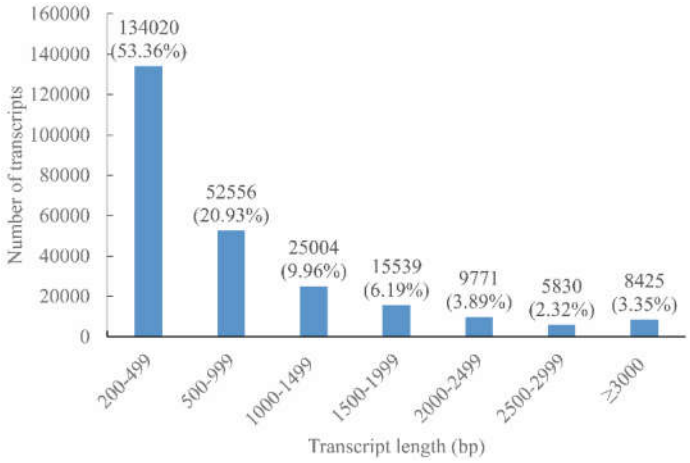
Sample/replicate	Raw reads	Clean reads	Clean bases (%)	Q20	GC%
Control 1-1	40,305,567	36,128,683	4.52G	95.35	54.76
Control 1-2	40,305,567	36,128,683	4.52G	92.29	54.7
Control 2-1	37,982,847	34,737,380	4.34G	95.34	55
Control 2-2	37,982,847	34,737,380	4.34G	92.4	54.93
Cold 1-1	35,495,938	32,200,406	4.03G	95.42	54.76
Cold 1-2	35,495,938	32,200,406	4.03G	92.39	54.68
Cold 2-1	41,113,853	35,916,262	4.49G	95.91	54.37
Cold 2-2	41,113,853	35,916,262	4.49G	92.82	54.32
Drought 1-1	38,734,285	35,137,981	4.39G	95.44	54.48
Drought 1-2	38,734,285	35,137,981	4.39G	92.5	54.41
Drought 2-1	36,736,709	32,995,519	4.12G	95.65	54.18
Drought 2-2	36,736,709	32,995,519	4.12G	92.4	54.11

The sample of this study contained 2 replications for 2 samples of three treatments.



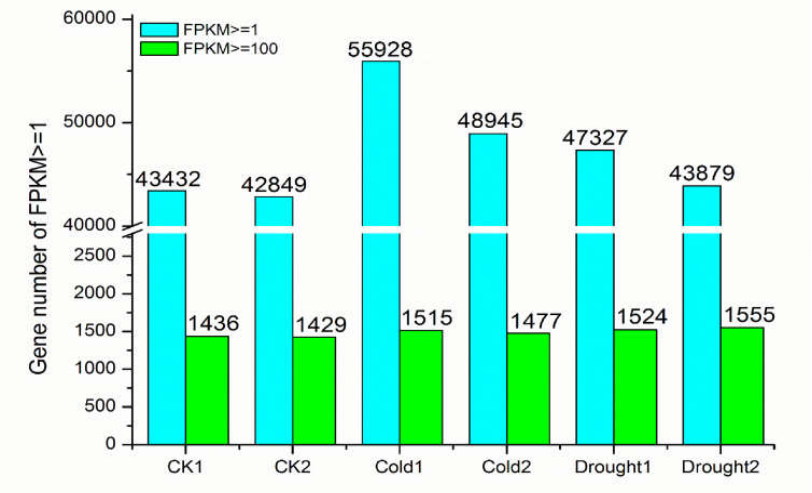
Size distribution of the unigene sequences.

The unigenes with different lengths (200 to 499 bp, 500 to 999 bp, 1000 to 1499 bp, 1500 to 1999 bp, 2000 to 2999 bp and ≥ 3000 bp) were shown, respectively. The percentages of the unigenes in each group out of the total unigenes (184,280) were also indicated.



Size distribution of the transcript sequences.

The transcripts with different lengths (200 to 499 bp, 500 to 999 bp, 1000 to 1499 bp, 1500 to 1999 bp, 2000 to 2999 bp and ≥ 3000 bp) were shown, respectively. The percentages of the transcripts in each group out of the total transcripts (251,145) were also indicated.



The number of genes with differential expression found in the *Zea mays* ssp. *mexicana* L. expression libraries of duplicate samples (FPKM ≥ 1).

Blue color means the number of genes of differential treatment with FPKM>=1; Green color means the number of genes differential treatment with FPKM>=100.

Supplemental file 2 GO classification of annotated unigenes.
http://journal.frontiersin.org/article/10.3389/fpls.2017.00136/full#supplementary-material

Supplemental file 3 Blast against maize and *Arabidopsis* protein database.
http://journal.frontiersin.org/article/10.3389/fpls.2017.00136/full#supplementary-material

Supplemental file 4 Differentially expressed genes in different samples.
http://journal.frontiersin.org/article/10.3389/fpls.2017.00136/full#supplementary-material

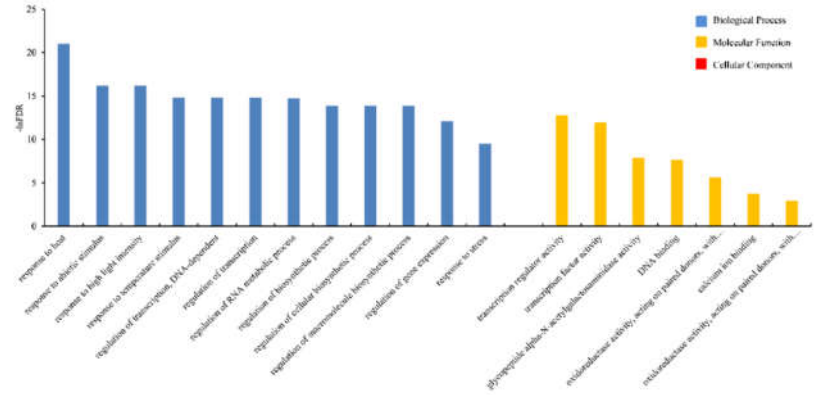
Supplemental file 5 GO enrichment analysis by direct comparing cold with drought stresses.

GO term ID	Term_type	Term description	FDR
GO:0009408	P	response to heat	4.00E-19
GO:0009266	P	response to temperature stimulus	1.90E-12
GO:0009628	P	response to abiotic stimulus	8.60E-11
GO:0006950	P	response to stress	8.60E-11
GO:0050896	P	response to stimulus	1.40E-06
GO:0042542	P	response to hydrogen peroxide	3.90E-06
GO:0000302	P	response to reactive oxygen species	5.70E-06
GO:0009644	P	response to high light intensity	3.20E-05
GO:0042221	P	response to chemical stimulus	4.30E-05
GO:0016052	P	carbohydrate catabolic process	5.00E-05
GO:0000272	P	polysaccharide catabolic process	7.30E-05
GO:0009415	P	response to water	0.00015
GO:0009414	P	response to water deprivation	0.00031
GO:0004722	F	protein serine/threonine phosphatase activity	0.00052
GO:0005983	P	starch catabolic process	0.00081
GO:0016161	F	beta-amylase activity	0.002
GO:0009251	P	glucan catabolic process	0.0029
GO:0009738	P	abscisic acid mediated signaling pathway	0.0029
GO:0006470	P	protein amino acid dephosphorylation	0.0029
GO:0016160	F	amylase activity	0.0036
GO:0006560	P	proline metabolic process	0.0037
GO:0006355	P	regulation of transcription, DNA-dependent	0.0061
GO:0009642	P	response to light intensity	0.0061
GO:0009615	P	response to virus	0.0061
GO:0045449	P	regulation of transcription	0.0061
GO:0051252	P	regulation of RNA metabolic process	0.0065
GO:0006350	P	transcription	0.0068
GO:0006351	P	transcription, DNA-dependent	0.0068
GO:0031326	P	regulation of cellular biosynthetic process	0.0068

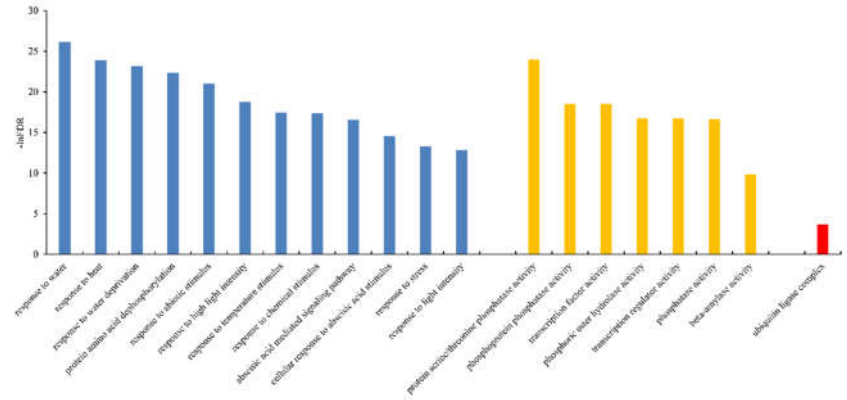
GO:0032774	P	RNA biosynthetic process	0.0072
GO:0009889	P	regulation of biosynthetic process	0.0072
GO:0006979	P	response to oxidative stress	0.0073
GO:0071215	P	cellular response to abscisic acid stimulus	0.0076
GO:0010556	P	regulation of macromolecule biosynthetic process	0.0082
GO:0080090	P	regulation of primary metabolic process	0.0086
GO:0010035	P	response to inorganic substance	0.0086
GO:0009737	P	response to abscisic acid stimulus	0.0086
GO:0019219	P	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.0086
GO:0009416	P	response to light stimulus	0.0089

Histogram of Gene Ontology (GO) enriched for differential expression unigenes. (A) Ck vs Cold, (B) Ck vs. Drought and (C) Cold vs. Drought. The X-axis represents the GO subcategories, and the Y-axis (-log10 FDR value) shows he degree of significant enrichment.

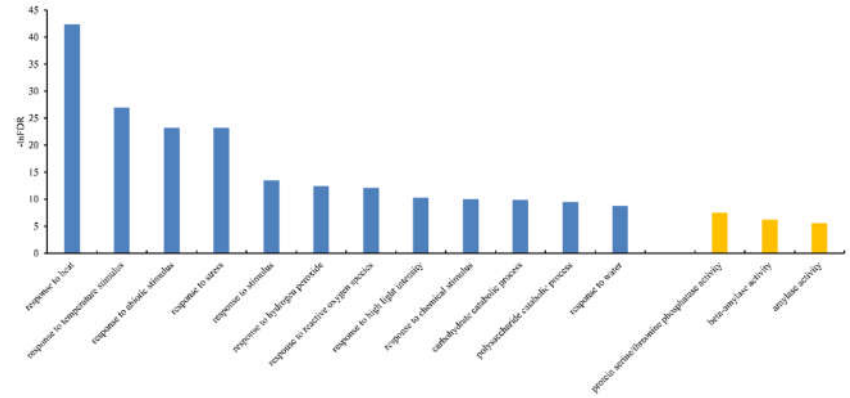
A



B



C



Supplemental file 6 Primers used in qRT-PCR.

Unigene ID	Primers	Description
c52482_g3	F:TCACGAACGGCTTCAGGTAG	protein phosphatase 2C 3 [*Arabidopsis thaliana*]/uncharacterized [*Zea mays*]
	R:TGCCTGCGTCGTTCTAATCA	
c61291_g1	F:TCCGTATCCATCCTTGTCCCT	PREDICTED: probable calcium-binding protein CML16 [*Zea mays*]
	R:AGTATTTGCTGGGGTGGCAG	
c59856_g7	F:ACGGCGAAGAACCGGATAAG	heat shock factor protein HSF30 [*Zea mays*]
	R:AAAAGAAGGCAAACCACACGG	
c57152_g1	F:TATTTCTTACCAATGTCGTCCCCAC	PREDICTED: transcription factor FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR-like
	R:TCACGTGCGCCATCTCTC	[*Zea mays*]
c51372_g1	F:GGGCCAGCCTAACACGAA	PREDICTED: CBL-interacting serine/threonine-protein kinase 11 isoform X1 [*Zea mays*]
	R:CAACAATAAACGGGTCGGCA	
c54045_g1	F:TGGAATGGAAGCGCAGACAC	PREDICTED: putative MYB DNA-binding domain superfamily protein isoform X1 [*Zea mays*]
	R:TTCATCCGTGGCTTTGCCTA	
c57309_g1	F:AGAACAAGAATTGCCCACGC	PREDICTED: E3 ubiquitin-protein ligase EL5-like [*Zea mays*]
	R:ACCTGCCAAGGATTTAGACGG	
c54089_g2	F:TCGTCCAGCTTTAGCCCTTC	heat shock protein 82 [*Zea mays*]
	R:GGCTACGAGGTGCTGTTCAT	
c56552_g1	F:TCGTCTGGGGTTTCGTTTGA	PREDICTED: 17.0 kDa class II heat shock protein [*Zea mays*]
	R:ACGTGGCATAACATGTCTCCTT	
c58118_g1	F:CGTATGGATAGGGCGTGAGG	PREDICTED: photosystem I reaction center subunit N, chloroplastic-like [*Zea mays*]
	R:GCAGACAGCCTAAGCAAGCA	
c58084_g2	F:GGACAAGGAGCGACTCTAAGC	PREDICTED: probable protein phosphatase 2C 37 [*Zea mays*]
	R:CAGAGACACAGGTAGCCACG	
c60754_g2	F:ACGTGTGGTGGATTCATGGT	homeobox-leucine zipper protein ATHB-6 [*Zea mays*]
	R:TCTGTTTCCCGTCGATCCCT	

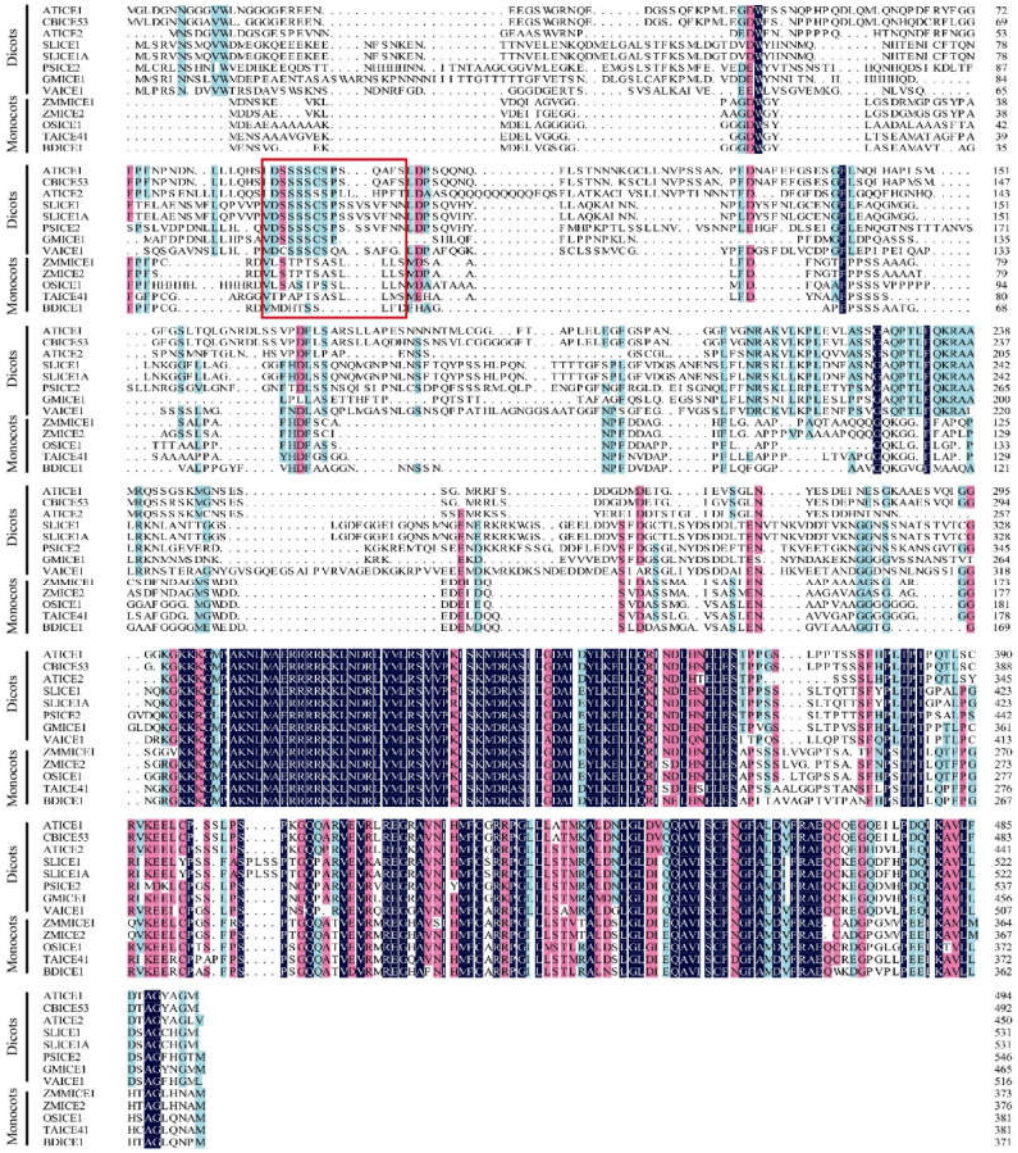
Supplemental file 7 GO enrichment analysis and classify of DEGs.
http://journal.frontiersin.org/article/10.3389/fpls.2017.00136/full#supplementary-material

Supplemental file 8 Growth phenotype.

WT | *ice1-2* mutant | CM 22:22 | CM 22:27

*Growth phenotype of three-week-old WT, *ice1-2* mutant and T3 homozygous transgenic plants CM 22:22/7.

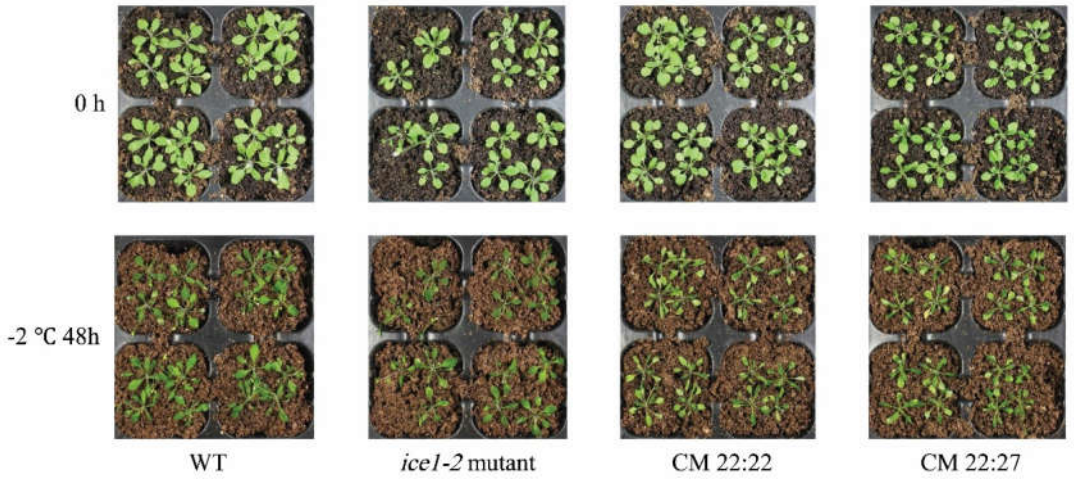
Supplemental file 9 Comparison of ICE-like genes amino acid sequences with homology proteins between part of dicots and monocots plants.



* The origin and GenBank accession numbers of compared sequences are as follows: *SlICE1, SlICEa* [*Solanum lycopersicum*] (AK247172, JX625139); *GmICE1* [*Glycine max*] (FJ393223); *AtICE1*, *AtICE2* [*Arabidopsis thaliana*] (AT3G26744, NP_172746); *VaICE1*[*Vitis amurensis*] (AGP04217) and *CbICE1*[*Capsella bursa-pastoris*] (AY506804); *PsICE2* [*Populus suaveolens*] (HM627255); *ZmICE2* [*Zea mays*] (ACG46593); *OsICE1* [*Oryza sativa Japonica* Group] (BAD88163); *BdICE1*[*Brachypodium distachyon*]

(XP_003567427); *TaICE41*[*Triticum aestivum*] (ACB69501). ClustalX 1.8 and DNAman 8.0 alignment were employed for deduced sequence homology alignment. Residues in black and gray regions indicate identical and similar residues, respectively, between isoforms. The red box showed the S-rich domain.

Supplemental file 10 Photos of physiological experiments.



*Photos of physiological experiments. There-week-old plants were treated at -2 ℃ for physiological experiments. Photographs were taken at 0 h and 48 h.

Supplemental file 11 *Fv/Fm* significance analysis.

		Average difference	SD
8493-25 ºC	You12-25 ºC	-.04400	.02171
	Inbred line-25 ºC	.04333	.02171
	8493-15 ºC	.00300	.02171
	You12-15 ºC	-.00533	.02171
	Inbred line-15 ºC	.00633	.02171
	8493-5 ºC	.08467*	.02171
	You12-5 ºC	.06500*	.02171
	Inbred line-5 ºC	.10467*	.02171
You12-25 ºc	8493-25 ºC	.04400	.02171
	Inbred line-25 ºC	.08733*	.02171
	8493-15 ºC	.04700*	.02171
	You12-15 ºC	.03867	.02171
	Inbred line-15 ºC	.05033*	.02171
	8493-5 ºC	.12867*	.02171
	You12-5 ºC	.10900*	.02171
	Inbred line-5 ºC	.14867*	.02171
Inbred line-25 ºC	8493-25 ºC	-.04333	.02171
	You12-25 ºC	-.08733*	.02171
	8493-15 ºC	-.04033	.02171
	You12-15 ºC	-.04867*	.02171
	Inbred line-15 ºC	-.03700	.02171
	8493-5 ºC	.04133	.02171
	You12-5 ºC	.02167	.02171
	Inbred line-5 ºC	.06133*	.02171
8493-15 ºC	8493-25 ºC	-.00300	.02171
	You12-25 ºC	-.04700*	.02171
	Inbred line-25 ºC	.04033	.02171
	You12-15 ºC	-.00833	.02171
	Inbred line-15 ºC	.00333	.02171
	8493-5 ºC	.08167*	.02171
	You12-5 ºC	.06200*	.02171
	Inbred line-5 ºC	.10167*	.02171
You12-15 ºC	8493-25 ºC	.00533	.02171
	You12-25 ºC	-.03867	.02171
	Inbred line-25 ºC	.04867*	.02171

	8493-15 ºC	.00833	.02171
	Inbred line-15 ºC	.01167	.02171
	8493-5 ºC	.09000*	.02171
	You12-5 ºC	.07033*	.02171
	Inbred line-5 ºC	.11000*	.02171
Inbred line-15 ºC	8493-25 ºC	-.00633	.02171
	You12-25 ºC	-.05033*	.02171
	Inbred line-25 ºC	.03700	.02171
	8493-15 ºC	-.00333	.02171
	You12-15 ºC	-.01167	.02171
	8493-5 ºC	.07833*	.02171
	You12-5 ºC	.05867*	.02171
	Inbred line-5 ºC	.09833*	.02171
8493-5 ºC	8493-25 ºC	-.08467*	.02171
	You12-25 ºC	-.12867*	.02171
	Inbred line-25 ºC	-.04133	.02171
	8493-15 ºC	-.08167*	.02171
	You12-15 ºC	-.09000*	.02171
	Inbred line-15 ºC	-.07833*	.02171
	You12-5 ºC	-.01967	.02171
	Inbred line-5 ºC	.02000	.02171
You12-5 ºC	8493-25 ºC	-.06500*	.02171
	You12-25 ºC	-.10900*	.02171
	Inbred line-25 ºC	-.02167	.02171
	8493-15 ºC	-.06200*	.02171
	You12-15 ºC	-.07033*	.02171
	Inbred line-15 ºC	-.05867*	.02171
	8493-5 ºC	.01967	.02171
	Inbred line-5 ºC	.03967	.02171
Inbred line-5 ºC	8493-25 ºC	-.10467*	.02171
	You12-25 ºC	-.14867*	.02171
	Inbred line-25 ºC	-.06133*	.02171
	8493-15 ºC	-.10167*	.02171
	You12-15 ºC	-.11000*	.02171
	Inbred line-15 ºC	-.09833*	.02171
	8493-5 ºC	-.02000	.02171

	You12-5 ºC	-.03967	.02171

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

Supplemental file 12 The Photochemical parameters of significance analysis.

***Fv'/Fm'* significance analysis**

		Average difference	SD
8493-25 ºC	You12-25 ºC	-.06067*	.02694
	Inbred line-25 ºC	.05467	.02694
	8493-15 ºC	.00633	.02694
	You12-15 ºC	-.00567	.02694
	Inbred line-15 ºC	.01933	.02694
	8493-5 ºC	0.053666667	.02694
	You12-5 ºC	0.031	.02694
	Inbred line-5 ºC	0.042	.02694
You12-25 ºc	8493-25 ºC	.06067*	.02694
	Inbred line-25 ºC	.11533*	.02694
	8493-15 ºC	.06700*	.02694
	You12-15 ºC	.05500	.02694
	Inbred line-15 ºC	.08000*	.02694
	8493-5 ºC	.11433*	.02694
	You12-5 ºC	.09167*	.02694
	Inbred line-5 ºC	.10267*	.02694
Inbred line-25 ºC	8493-25 ºC	-.05467	.02694
	You12-25 ºC	-.11533*	.02694
	8493-15 ºC	-.04833	.02694
	You12-15 ºC	-.06033*	.02694
	Inbred line-15 ºC	-.03533	.02694
	8493-5 ºC	-.00100	.02694
	You12-5 ºC	-.02367	.02694
	Inbred line-5 ºC	-0.012666667	.02694
8493-15 ºC	8493-25 ºC	-.00633	.02694
	You12-25 ºC	-.06700*	.02694
	Inbred line-25 ºC	.04833	.02694
	You12-15 ºC	-.01200	.02694
	Inbred line-15 ºC	.01300	.02694
	8493-5 ºC	0.047333333	.02694
	You12-5 ºC	0.024666667	.02694
	Inbred line-5 ºC	0.035666667	.02694

You12-15 ºC	8493-25 ºC	.00567	.02694
	You12-25 ºC	-.05500	.02694
	Inbred line-25 ºC	.06033*	.02694
	8493-15 ºC	.01200	.02694
	Inbred line-15 ºC	.02500	.02694
	8493-5 ºC	.05933*	.02694
	You12-5 ºC	0.036666667	.02694
	Inbred line-5 ºC	0.047666667	.02694
Inbred line-15 ºC	8493-25 ºC	-.01933	.02694
	You12-25 ºC	-.08000*	.02694
	Inbred line-25 ºC	.03533	.02694
	8493-15 ºC	-.01300	.02694
	You12-15 ºC	-.02500	.02694
	8493-5 ºC	0.034333333	.02694
	You12-5 ºC	0.011666667	.02694
	Inbred line-5 ºC	0.022666667	.02694
8493-5 ºC	8493-25 ºC	-0.053666667	.02694
	You12-25 ºC	-.11433*	.02694
	Inbred line-25 ºC	.00100	.02694
	8493-15 ºC	-0.047333333	.02694
	You12-15 ºC	-.05933*	.02694
	Inbred line-15 ºC	-0.034333333	.02694
	You12-5 ºC	-.02267	.02694
	Inbred line-5 ºC	-.01167	.02694
You12-5 ºC	8493-25 ºC	-0.031	.02694
	You12-25 ºC	-.09167*	.02694
	Inbred line-25 ºC	.02367	.02694
	8493-15 ºC	-0.024666667	.02694
	You12-15 ºC	-0.036666667	.02694
	Inbred line-15 ºC	-0.011666667	.02694
	8493-5 ºC	.02267	.02694
	Inbred line-5 ºC	.01100	.02694
Inbred line-5 ºC	8493-25 ºC	-0.042	.02694
	You12-25 ºC	-.10267*	.02694
	Inbred line-25 ºC	0.012666667	.02694
	8493-15 ºC	-0.035666667	.02694
	You12-15 ºC	-0.047666667	.02694
	Inbred line-15 ºC	-0.022666667	.02694
	8493-5 ºC	.01167	.02694
	You12-5 ºC	-.01100	.02694

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

SPII significance analysis

		Average difference	SD
8493-25 ºC	You12-25 ºC	-.0166667*	.00726
	Inbred line-25 ºC	-.00433	.00726
	8493-15 ºC	-.00967	.00726
	You12-15 ºC	-.00400	.00726
	Inbred line-15 ºC	-.01033	.00726
	8493-5 ºC	.0576667*	.00726
	You12-5 ºC	.0370000*	.00726
	Inbred line-5 ºC	.0310000*	.00726
You12-25 ºc	8493-25 ºC	.0166667*	.00726
	Inbred line-25 ºC	0.012333333	.00726
	8493-15 ºC	0.007	.00726
	You12-15 ºC	.01267	.00726
	Inbred line-15 ºC	0.006333333	.00726
	8493-5 ºC	.0743333*	.00726
	You12-5 ºC	.0536667*	.00726
	Inbred line-5 ºC	.0476667*	.00726
Inbred line-25 ºC	8493-25 ºC	.00433	.00726
	You12-25 ºC	-0.012333333	.00726
	8493-15 ºC	-.00533	.00726
	You12-15 ºC	0.000333333	.00726
	Inbred line-15 ºC	-.00600	.00726
	8493-5 ºC	.0620000*	.00726
	You12-5 ºC	.0413333*	.00726
	Inbred line-5 ºC	.0353333*	.00726
8493-15 ºC	8493-25 ºC	.00967	.00726
	You12-25 ºC	-0.007	.00726
	Inbred line-25 ºC	.00533	.00726
	You12-15 ºC	.00567	.00726
	Inbred line-15 ºC	-.00067	.00726
	8493-5 ºC	.0673333*	.00726
	You12-5 ºC	.0466667*	.00726
	Inbred line-5 ºC	.0406667*	.00726
You12-15 ºC	8493-25 ºC	.00400	.00726
	You12-25 ºC	-.01267	.00726
	Inbred line-25 ºC	-0.000333333	.00726

	8493-15 ºC	-.00567	.00726
	Inbred line-15 ºC	-.00633	.00726
	8493-5 ºC	.0616667*	.00726
	You12-5 ºC	.0410000*	.00726
	Inbred line-5 ºC	.0350000*	.00726
Inbred line-15 ºC	8493-25 ºC	.01033	.00726
	You12-25 ºC	-0.006333333	.00726
	Inbred line-25 ºC	.00600	.00726
	8493-15 ºC	.00067	.00726
	You12-15 ºC	.00633	.00726
	8493-5 ºC	.0680000*	.00726
	You12-5 ºC	.0473333*	.00726
	Inbred line-5 ºC	.0413333*	.00726
8493-5 ºC	8493-25 ºC	-.0576667*	.00726
	You12-25 ºC	-.0743333*	.00726
	Inbred line-25 ºC	-.0620000*	.00726
	8493-15 ºC	-.0673333*	.00726
	You12-15 ºC	-.0616667*	.00726
	Inbred line-15 ºC	-.0680000*	.00726
	You12-5 ºC	-.0206667*	.00726
	Inbred line-5 ºC	-.0266667*	.00726
You12-5 ºC	8493-25 ºC	-.0370000*	.00726
	You12-25 ºC	-.0536667*	.00726
	Inbred line-25 ºC	-.0413333*	.00726
	8493-15 ºC	-.0466667*	.00726
	You12-15 ºC	-.0410000*	.00726
	Inbred line-15 ºC	-.0473333*	.00726
	8493-5 ºC	.0206667*	.00726
	Inbred line-5 ºC	-.00600	.00726
Inbred line-5 ºC	8493-25 ºC	-.0310000*	.00726
	You12-25 ºC	-.0476667*	.00726
	Inbred line-25 ºC	-.0353333*	.00726
	8493-15 ºC	-.0406667*	.00726
	You12-15 ºC	-.0350000*	.00726
	Inbred line-15 ºC	-.0413333*	.00726
	8493-5 ºC	.0266667*	.00726
	You12-5 ºC	.00600	.00726

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

qP significance analysis

		Average difference	SD
8493-25 ºC	You12-25 ºC	.01100	.01103
	Inbred line-25 ºC	-.0486667*	.01103
	8493-15 ºC	.1306667*	.01103
	You12-15 ºC	.1363333*	.01103
	Inbred line-15 ºC	.1300000*	.01103
	8493-5 ºC	.1980000*	.01103
	You12-5 ºC	.1773333*	.01103
	Inbred line-5 ºC	.1713333*	.01103
You12-25 ºc	8493-25 ºC	-.01100	.01103
	Inbred line-25 ºC	-.0596667*	.01103
	8493-15 ºC	.1196667*	.01103
	You12-15 ºC	.1253333*	.01103
	Inbred line-15 ºC	.1190000*	.01103
	8493-5 ºC	.1870000*	.01103
	You12-5 ºC	.1663333*	.01103
	Inbred line-5 ºC	.1603333*	.01103
Inbred line-25 ºC	8493-25 ºC	.0486667*	.01103
	You12-25 ºC	.0596667*	.01103
	8493-15 ºC	.1793333*	.01103
	You12-15 ºC	.1850000*	.01103
	Inbred line-15 ºC	.1786667*	.01103
	8493-5 ºC	.2466667*	.01103
	You12-5 ºC	.2260000*	.01103
	Inbred line-5 ºC	.2200000*	.01103
8493-15 ºC	8493-25 ºC	-.1306667*	.01103
	You12-25 ºC	-.1196667*	.01103
	Inbred line-25 ºC	-.1793333*	.01103
	You12-15 ºC	.00567	.01103
	Inbred line-15 ºC	-.00067	.01103
	8493-5 ºC	.0673333*	.01103
	You12-5 ºC	.0466667*	.01103
	Inbred line-5 ºC	.0406667*	.01103
You12-15 ºC	8493-25 ºC	-.1363333*	.01103
	You12-25 ºC	-.1253333*	.01103
	Inbred line-25 ºC	-.1850000*	.01103
	8493-15 ºC	-.00567	.01103
	Inbred line-15 ºC	-.00633	.01103
	8493-5 ºC	.0616667*	.01103

	You12-5 ºC	.0410000*	.01103
	Inbred line-5 ºC	.0350000*	.01103
Inbred line-15 ºC	8493-25 ºC	-.1300000*	.01103
	You12-25 ºC	-.1190000*	.01103
	Inbred line-25 ºC	-.1786667*	.01103
	8493-15 ºC	.00067	.01103
	You12-15 ºC	.00633	.01103
	8493-5 ºC	.0680000*	.01103
	You12-5 ºC	.0473333*	.01103
	Inbred line-5 ºC	.0413333*	.01103
8493-5 ºC	8493-25 ºC	-.1980000*	.01103
	You12-25 ºC	-.1870000*	.01103
	Inbred line-25 ºC	-.2466667*	.01103
	8493-15 ºC	-.0673333*	.01103
	You12-15 ºC	-.0616667*	.01103
	Inbred line-15 ºC	-.0680000*	.01103
	You12-5 ºC	-.02067	.01103
	Inbred line-5 ºC	-.0266667*	.01103
You12-5 ºC	8493-25 ºC	-.1773333*	.01103
	You12-25 ºC	-.1663333*	.01103
	Inbred line-25 ºC	-.2260000*	.01103
	8493-15 ºC	-.0466667*	.01103
	You12-15 ºC	-.0410000*	.01103
	Inbred line-15 ºC	-.0473333*	.01103
	8493-5 ºC	.02067	.01103
	Inbred line-5 ºC	-.00600	.01103
Inbred line-5 ºC	8493-25 ºC	-.1713333*	.01103
	You12-25 ºC	-.1603333*	.01103
	Inbred line-25 ºC	-.2200000*	.01103
	8493-15 ºC	-.0406667*	.01103
	You12-15 ºC	-.0350000*	.01103
	Inbred line-15 ºC	-.0413333*	.01103
	8493-5 ºC	.0266667*	.01103
	You12-5 ºC	.00600	.01103

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

NPQ significance analysis

		Average difference	SD
8493-25 ºC	You12-25 ºC	.03100	.25309
	Inbred line-25 ºC	-.32867	.25309
	8493-15 ºC	-.10767	.25309
	You12-15 ºC	-.06200	.25309
	Inbred line-15 ºC	-.21433	.25309
	8493-5 ºC	-0.355333333	.25309
	You12-5 ºC	-0.349	.25309
	Inbred line-5 ºC	-.5993333*	.25309
You12-25 ºc	8493-25 ºC	-.03100	.25309
	Inbred line-25 ºC	-0.359666667	.25309
	8493-15 ºC	-0.138666667	.25309
	You12-15 ºC	-.09300	.25309
	Inbred line-15 ºC	-0.245333333	.25309
	8493-5 ºC	-0.386333333	.25309
	You12-5 ºC	-0.38	.25309
	Inbred line-5 ºC	-.6303333*	.25309
Inbred line-25 ºC	8493-25 ºC	.32867	.25309
	You12-25 ºC	0.359666667	.25309
	8493-15 ºC	.22100	.25309
	You12-15 ºC	0.266666667	.25309
	Inbred line-15 ºC	.11433	.25309
	8493-5 ºC	-.02667	.25309
	You12-5 ºC	-.02033	.25309
	Inbred line-5 ºC	-0.270666667	.25309
8493-15 ºC	8493-25 ºC	.10767	.25309
	You12-25 ºC	0.138666667	.25309
	Inbred line-25 ºC	-.22100	.25309
	You12-15 ºC	.04567	.25309
	Inbred line-15 ºC	-.10667	.25309
	8493-5 ºC	-0.247666667	.25309
	You12-5 ºC	-0.241333333	.25309
	Inbred line-5 ºC	-0.491666667	.25309
You12-15 ºC	8493-25 ºC	.06200	.25309
	You12-25 ºC	.09300	.25309
	Inbred line-25 ºC	-0.266666667	.25309
	8493-15 ºC	-.04567	.25309
	Inbred line-15 ºC	-.15233	.25309
	8493-5 ºC	-0.293333333	.25309

	You12-5 ºC	-0.287	.25309
	Inbred line-5 ºC	-.5373333*	.25309
Inbred line-15 ºC	8493-25 ºC	.21433	.25309
	You12-25 ºC	0.245333333	.25309
	Inbred line-25 ºC	-.11433	.25309
	8493-15 ºC	.10667	.25309
	You12-15 ºC	.15233	.25309
	8493-5 ºC	-0.141	.25309
	You12-5 ºC	-0.134666667	.25309
	Inbred line-5 ºC	-0.385	.25309
8493-5 ºC	8493-25 ºC	0.355333333	.25309
	You12-25 ºC	0.386333333	.25309
	Inbred line-25 ºC	.02667	.25309
	8493-15 ºC	0.247666667	.25309
	You12-15 ºC	0.293333333	.25309
	Inbred line-15 ºC	0.141	.25309
	You12-5 ºC	.00633	.25309
	Inbred line-5 ºC	-.24400	.25309
You12-5 ºC	8493-25 ºC	0.349	.25309
	You12-25 ºC	0.38	.25309
	Inbred line-25 ºC	.02033	.25309
	8493-15 ºC	0.241333333	.25309
	You12-15 ºC	0.287	.25309
	Inbred line-15 ºC	0.134666667	.25309
	8493-5 ºC	-.00633	.25309
	Inbred line-5 ºC	-.25033	.25309
Inbred line-5 ºC	8493-25 ºC	.5993333*	.25309
	You12-25 ºC	.6303333*	.25309
	Inbred line-25 ºC	0.270666667	.25309
	8493-15 ºC	0.491666667	.25309
	You12-15 ºC	.5373333*	.25309
	Inbred line-15 ºC	0.385	.25309
	8493-5 ºC	.24400	.25309
	You12-5 ºC	.25033	.25309

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

Supplemental file 13 Dynamic values of significance analysis.
qP dynamic values of significance analysis.

		Average difference	SD
8493-25 ºC	You12-25 ºC	.0526667*	.01859
	Inbred line-25 ºC	.0810000*	.01859
	8493-15 ºC	.00000	.01859
	You12-15 ºC	.03800	.01859
	Inbred line-15 ºC	.02167	.01859
	8493-5 ºC	0	.01859
	You12-5 ºC	.0413333*	.01859
	Inbred line-5 ºC	.0776667*	.01859
You12-25 ºc	8493-25 ºC	-.0526667*	.01859
	Inbred line-25 ºC	0.028333333	.01859
	8493-15 ºC	-.0526667*	.01859
	You12-15 ºC	-.01467	.01859
	Inbred line-15 ºC	-0.031	.01859
	8493-5 ºC	-.0526667*	.01859
	You12-5 ºC	-0.011333333	.01859
	Inbred line-5 ºC	0.025	.01859
Inbred line-25 ºC	8493-25 ºC	-.0810000*	.01859
	You12-25 ºC	-0.028333333	.01859
	8493-15 ºC	-.0810000*	.01859
	You12-15 ºC	-.0430000*	.01859
	Inbred line-15 ºC	-.0593333*	.01859
	8493-5 ºC	-.0810000*	.01859
	You12-5 ºC	-.0396667*	.01859
	Inbred line-5 ºC	-0.003333333	.01859
8493-15 ºC	8493-25 ºC	.00000	.01859
	You12-25 ºC	.0526667*	.01859
	Inbred line-25 ºC	.0810000*	.01859
	You12-15 ºC	.03800	.01859
	Inbred line-15 ºC	.02167	.01859
	8493-5 ºC	0	.01859
	You12-5 ºC	.0413333*	.01859
	Inbred line-5 ºC	.0776667*	.01859
You12-15 ºC	8493-25 ºC	-.03800	.01859
	You12-25 ºC	.01467	.01859
	Inbred line-25 ºC	.0430000*	.01859
	8493-15 ºC	-.03800	.01859
	Inbred line-15 ºC	-.01633	.01859

	8493-5 ºC	-0.038	.01859
	You12-5 ºC	0.003333333	.01859
	Inbred line-5 ºC	.0396667*	.01859
Inbred line-15 ºC	8493-25 ºC	-.02167	.01859
	You12-25 ºC	0.031	.01859
	Inbred line-25 ºC	.0593333*	.01859
	8493-15 ºC	-.02167	.01859
	You12-15 ºC	.01633	.01859
	8493-5 ºC	-0.021666667	.01859
	You12-5 ºC	0.019666667	.01859
	Inbred line-5 ºC	.0560000*	.01859
8493-5 ºC	8493-25 ºC	0	.01859
	You12-25 ºC	.0526667*	.01859
	Inbred line-25 ºC	.0810000*	.01859
	8493-15 ºC	0	.01859
	You12-15 ºC	0.038	.01859
	Inbred line-15 ºC	0.021666667	.01859
	You12-5 ºC	.0413333*	.01859
	Inbred line-5 ºC	.0776667*	.01859
You12-5 ºC	8493-25 ºC	-.0413333*	.01859
	You12-25 ºC	0.011333333	.01859
	Inbred line-25 ºC	.0396667*	.01859
	8493-15 ºC	-.0413333*	.01859
	You12-15 ºC	-0.003333333	.01859
	Inbred line-15 ºC	-0.019666667	.01859
	8493-5 ºC	-.0413333*	.01859
	Inbred line-5 ºC	.03633	.01859
Inbred line-5 ºC	8493-25 ºC	-.0776667*	.01859
	You12-25 ºC	-0.025	.01859
	Inbred line-25 ºC	0.003333333	.01859
	8493-15 ºC	-.0776667*	.01859
	You12-15 ºC	-.0396667*	.01859
	Inbred line-15 ºC	-.0560000*	.01859
	8493-5 ºC	-.0776667*	.01859
	You12-5 ºC	-.03633	.01859

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

NPQ dynamic values of significance analysis.

		Average difference	SD
8493-25 ºC	You12-25 ºC	-.4073333*	.18533
	Inbred line-25 ºC	-.4416667*	.18533
	8493-15 ºC	.00000	.18533
	You12-15 ºC	-.22200	.18533
	Inbred line-15 ºC	-.24933	.18533
	8493-5 ºC	0	.18533
	You12-5 ºC	-0.245333333	.18533
	Inbred line-5 ºC	.3903333*	.18533
You12-25 ºc	8493-25 ºC	.4073333*	.18533
	Inbred line-25 ºC	-0.034333333	.18533
	8493-15 ºC	.4073333*	.18533
	You12-15 ºC	.18533	.18533
	Inbred line-15 ºC	0.158	.18533
	8493-5 ºC	.4073333*	.18533
	You12-5 ºC	0.162	.18533
	Inbred line-5 ºC	.7976667*	.18533
Inbred line-25 ºC	8493-25 ºC	.4416667*	.18533
	You12-25 ºC	0.034333333	.18533
	8493-15 ºC	.4416667*	.18533
	You12-15 ºC	0.219666667	.18533
	Inbred line-15 ºC	.19233	.18533
	8493-5 ºC	.4416667*	.18533
	You12-5 ºC	.19633	.18533
	Inbred line-5 ºC	.8320000*	.18533
8493-15 ºC	8493-25 ºC	.00000	.18533
	You12-25 ºC	-.4073333*	.18533
	Inbred line-25 ºC	-.4416667*	.18533
	You12-15 ºC	-.22200	.18533
	Inbred line-15 ºC	-.24933	.18533
	8493-5 ºC	0	.18533
	You12-5 ºC	-0.245333333	.18533
	Inbred line-5 ºC	.3903333*	.18533
You12-15 ºC	8493-25 ºC	.22200	.18533
	You12-25 ºC	-.18533	.18533
	Inbred line-25 ºC	-0.219666667	.18533
	8493-15 ºC	.22200	.18533
	Inbred line-15 ºC	-.02733	.18533
	8493-5 ºC	0.222	.18533

	You12-5 ºC	-0.023333333	.18533
	Inbred line-5 ºC	.6123333*	.18533
Inbred line-15 ºC	8493-25 ºC	.24933	.18533
	You12-25 ºC	-0.158	.18533
	Inbred line-25 ºC	-.19233	.18533
	8493-15 ºC	.24933	.18533
	You12-15 ºC	.02733	.18533
	8493-5 ºC	0.249333333	.18533
	You12-5 ºC	0.004	.18533
	Inbred line-5 ºC	.6396667*	.18533
8493-5 ºC	8493-25 ºC	0	.18533
	You12-25 ºC	-.4073333*	.18533
	Inbred line-25 ºC	-.4416667*	.18533
	8493-15 ºC	0	.18533
	You12-15 ºC	-0.222	.18533
	Inbred line-15 ºC	-0.249333333	.18533
	You12-5 ºC	-.24533	.18533
	Inbred line-5 ºC	.3903333*	.18533
You12-5 ºC	8493-25 ºC	0.245333333	.18533
	You12-25 ºC	-0.162	.18533
	Inbred line-25 ºC	-.19633	.18533
	8493-15 ºC	0.245333333	.18533
	You12-15 ºC	0.023333333	.18533
	Inbred line-15 ºC	-0.004	.18533
	8493-5 ºC	.24533	.18533
	Inbred line-5 ºC	.6356667*	.18533
Inbred line-5 ºC	8493-25 ºC	-.3903333*	.18533
	You12-25 ºC	-.7976667*	.18533
	Inbred line-25 ºC	-.8320000*	.18533
	8493-15 ºC	-.3903333*	.18533
	You12-15 ºC	-.6123333*	.18533
	Inbred line-15 ºC	-.6396667*	.18533
	8493-5 ºC	-.3903333*	.18533
	You12-5 ºC	-.6356667*	.18533

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

Supplemental file 14 ETR significance analysis.

		Average difference	SD
8493-25 ºC	You12-25 ºC	33.1800000*	11.71366
	Inbred line-25 ºC	51.0300000*	11.71366
	8493-15 ºC	.00000	11.71366
	You12-15 ºC	23.94000	11.71366
	Inbred line-15 ºC	13.65000	11.71366
	8493-5 ºC	0	11.71366
	You12-5 ºC	26.0400000*	11.71366
	Inbred line-5 ºC	48.9300000*	11.71366
You12-25 ºc	8493-25 ºC	-33.1800000*	11.71366
	Inbred line-25 ºC	17.85	11.71366
	8493-15 ºC	-33.1800000*	11.71366
	You12-15 ºC	-9.24000	11.71366
	Inbred line-15 ºC	-19.53	11.71366
	8493-5 ºC	-33.1800000*	11.71366
	You12-5 ºC	-7.14	11.71366
	Inbred line-5 ºC	15.75	11.71366
Inbred line-25 ºC	8493-25 ºC	-51.0300000*	11.71366
	You12-25 ºC	-17.85	11.71366
	8493-15 ºC	-51.0300000*	11.71366
	You12-15 ºC	-27.0900000*	11.71366
	Inbred line-15 ºC	-37.3800000*	11.71366
	8493-5 ºC	-51.0300000*	11.71366
	You12-5 ºC	-24.9900000*	11.71366
	Inbred line-5 ºC	-2.1	11.71366
8493-15 ºC	8493-25 ºC	.00000	11.71366
	You12-25 ºC	33.1800000*	11.71366
	Inbred line-25 ºC	51.0300000*	11.71366
	You12-15 ºC	23.94000	11.71366
	Inbred line-15 ºC	13.65000	11.71366
	8493-5 ºC	0	11.71366
	You12-5 ºC	26.0400000*	11.71366
	Inbred line-5 ºC	48.9300000*	11.71366
You12-15 ºC	8493-25 ºC	-23.94000	11.71366
	You12-25 ºC	9.24000	11.71366
	Inbred line-25 ºC	27.0900000*	11.71366
	8493-15 ºC	-23.94000	11.71366
	Inbred line-15 ºC	-10.29000	11.71366
	8493-5 ºC	-23.94	11.71366

	You12-5 ºC	2.1	11.71366
	Inbred line-5 ºC	24.9900000*	11.71366
Inbred line-15 ºC	8493-25 ºC	-13.65000	11.71366
	You12-25 ºC	19.53	11.71366
	Inbred line-25 ºC	37.3800000*	11.71366
	8493-15 ºC	-13.65000	11.71366
	You12-15 ºC	10.29000	11.71366
	8493-5 ºC	-13.65	11.71366
	You12-5 ºC	12.39	11.71366
	Inbred line-5 ºC	35.2800000*	11.71366
8493-5 ºC	8493-25 ºC	0	11.71366
	You12-25 ºC	33.1800000*	11.71366
	Inbred line-25 ºC	51.0300000*	11.71366
	8493-15 ºC	0	11.71366
	You12-15 ºC	23.94	11.71366
	Inbred line-15 ºC	13.65	11.71366
	You12-5 ºC	26.0400000*	11.71366
	Inbred line-5 ºC	48.9300000*	11.71366
You12-5 ºC	8493-25 ºC	-26.0400000*	11.71366
	You12-25 ºC	7.14	11.71366
	Inbred line-25 ºC	24.9900000*	11.71366
	8493-15 ºC	-26.0400000*	11.71366
	You12-15 ºC	-2.1	11.71366
	Inbred line-15 ºC	-12.39	11.71366
	8493-5 ºC	-26.0400000*	11.71366
	Inbred line-5 ºC	22.89000	11.71366
Inbred line-5 ºC	8493-25 ºC	-48.9300000*	11.71366
	You12-25 ºC	-15.75	11.71366
	Inbred line-25 ºC	2.1	11.71366
	8493-15 ºC	-48.9300000*	11.71366
	You12-15 ºC	-24.9900000*	11.71366
	Inbred line-15 ºC	-35.2800000*	11.71366
	8493-5 ºC	-48.9300000*	11.71366
	You12-5 ºC	-22.89000	11.71366

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

Supplemental file 15 The content of Chl significance analysis.
Chl a significance analysis

		Average difference	SD
8493-25 °C	You12-25 ℃	.04780	.15378
	Inbred line-25 ℃	.21951	.15378
	8493-15 ℃	.15111	.15378
	You12-15 ℃	.09904	.15378
	Inbred line-15 ℃	.24269	.15378
	8493-5 ℃	-.3557166*	.15378
	You12-5 ℃	-0.105129133	.15378
	Inbred line-5 ℃	-0.120012633	.15378
You12-25 ℃	8493-25 ℃	-.04780	.15378
	Inbred line-25 ℃	0.171705333	.15378
	8493-15 ℃	0.1033091	.15378
	You12-15 ℃	.05124	.15378
	Inbred line-15 ℃	0.194881933	.15378
	8493-5 ℃	-.4035201*	.15378
	You12-5 ℃	-0.152932633	.15378
	Inbred line-5 ℃	-0.167816133	.15378
Inbred line-25 ℃	8493-25 ℃	-.21951	.15378
	You12-25 ℃	-0.171705333	.15378
	8493-15 ℃	-.06840	.15378
	You12-15 ℃	-0.120467567	.15378
	Inbred line-15 ℃	.02318	.15378
	8493-5 ℃	-.5752254*	.15378
	You12-5 ℃	-.3246380*	.15378
	Inbred line-5 ℃	-.3395215*	.15378
8493-15 ℃	8493-25 ℃	-.15111	.15378
	You12-25 ℃	-0.1033091	.15378
	Inbred line-25 ℃	.06840	.15378
	You12-15 ℃	-.05207	.15378
	Inbred line-15 ℃	.09157	.15378
	8493-5 ℃	-.5068292*	.15378
	You12-5 ℃	-0.256241733	.15378
	Inbred line-5 ℃	-0.271125233	.15378
You12-15 ℃	8493-25 ℃	-.09904	.15378
	You12-25 ℃	-.05124	.15378
	Inbred line-25 ℃	0.120467567	.15378
	8493-15 ℃	.05207	.15378
	Inbred line-15 ℃	.14364	.15378

	8493-5 ºC	-.4547579*	.15378
	You12-5 ºC	-0.2041704	.15378
	Inbred line-5 ºC	-0.2190539	.15378
Inbred line-15 ºC	8493-25 ºC	-.24269	.15378
	You12-25 ºC	-0.194881933	.15378
	Inbred line-25 ºC	-.02318	.15378
	8493-15 ºC	-.09157	.15378
	You12-15 ºC	-.14364	.15378
	8493-5 ºC	-.5984020*	.15378
	You12-5 ºC	-.3478146*	.15378
	Inbred line-5 ºC	-.3626981*	.15378
8493-5 ºC	8493-25 ºC	.3557166*	.15378
	You12-25 ºC	.4035201*	.15378
	Inbred line-25 ºC	.5752254*	.15378
	8493-15 ºC	.5068292*	.15378
	You12-15 ºC	.4547579*	.15378
	Inbred line-15 ºC	.5984020*	.15378
	You12-5 ºC	.25059	.15378
	Inbred line-5 ºC	.23570	.15378
You12-5 ºC	8493-25 ºC	0.105129133	.15378
	You12-25 ºC	0.152932633	.15378
	Inbred line-25 ºC	.3246380*	.15378
	8493-15 ºC	0.256241733	.15378
	You12-15 ºC	0.2041704	.15378
	Inbred line-15 ºC	.3478146*	.15378
	8493-5 ºC	-.25059	.15378
	Inbred line-5 ºC	-.01488	.15378
Inbred line-5 ºC	8493-25 ºC	0.120012633	.15378
	You12-25 ºC	0.167816133	.15378
	Inbred line-25 ºC	.3395215*	.15378
	8493-15 ºC	0.271125233	.15378
	You12-15 ºC	0.2190539	.15378
	Inbred line-15 ºC	.3626981*	.15378
	8493-5 ºC	-.23570	.15378
	You12-5 ºC	.01488	.15378

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

Chl b significance analysis

		Average difference	SD
8493-25 °C	You12-25 ºC	.01196	.06313
	Inbred line-25 ºC	.08452	.06313
	8493-15 ºC	.06743	.06313
	You12-15 ºC	.04353	.06313
	Inbred line-15 ºC	.09466	.06313
	8493-5 ºC	-.1610333*	.06313
	You12-5 ºC	-0.0394006	.06313
	Inbred line-5 ºC	-0.048886933	.06313
You12-25 ºc	8493-25 ºC	-.01196	.06313
	Inbred line-25 ºC	0.072560033	.06313
	8493-15 ºC	0.0554683	.06313
	You12-15 ºC	.03157	.06313
	Inbred line-15 ºC	0.082702633	.06313
	8493-5 ºC	-.1729943*	.06313
	You12-5 ºC	-0.051361633	.06313
	Inbred line-5 ºC	-0.060847967	.06313
Inbred line-25 ºC	8493-25 ºC	-.08452	.06313
	You12-25 ºC	-0.072560033	.06313
	8493-15 ºC	-.01709	.06313
	You12-15 ºC	-0.0409904	.06313
	Inbred line-15 ºC	.01014	.06313
	8493-5 ºC	-.2455543*	.06313
	You12-5 ºC	-.12392	.06313
	Inbred line-5 ºC	-.1334080*	.06313
8493-15 ºC	8493-25 ºC	-.06743	.06313
	You12-25 ºC	-0.0554683	.06313
	Inbred line-25 ºC	.01709	.06313
	You12-15 ºC	-.02390	.06313
	Inbred line-15 ºC	.02723	.06313
	8493-5 ºC	-.2284626*	.06313
	You12-5 ºC	-0.106829933	.06313
	Inbred line-5 ºC	-0.116316267	.06313
You12-15 ºC	8493-25 ºC	-.04353	.06313
	You12-25 ºC	-.03157	.06313
	Inbred line-25 ºC	0.0409904	.06313
	8493-15 ºC	.02390	.06313
	Inbred line-15 ºC	.05113	.06313
	8493-5 ºC	-.2045639*	.06313

	You12-5 ºC	-0.082931267	.06313
	Inbred line-5 ºC	-0.0924176	.06313
Inbred line-15 ºC	8493-25 ºC	-.09466	.06313
	You12-25 ºC	-0.082702633	.06313
	Inbred line-25 ºC	-.01014	.06313
	8493-15 ºC	-.02723	.06313
	You12-15 ºC	-.05113	.06313
	8493-5 ºC	-.2556969*	.06313
	You12-5 ºC	-.1340643*	.06313
	Inbred line-5 ºC	-.1435506*	.06313
8493-5 ºC	8493-25 ºC	.1610333*	.06313
	You12-25 ºC	.1729943*	.06313
	Inbred line-25 ºC	.2455543*	.06313
	8493-15 ºC	.2284626*	.06313
	You12-15 ºC	.2045639*	.06313
	Inbred line-15 ºC	.2556969*	.06313
	You12-5 ºC	.12163	.06313
	Inbred line-5 ºC	.11215	.06313
You12-5 ºC	8493-25 ºC	0.0394006	.06313
	You12-25 ºC	0.051361633	.06313
	Inbred line-25 ºC	.12392	.06313
	8493-15 ºC	0.106829933	.06313
	You12-15 ºC	0.082931267	.06313
	Inbred line-15 ºC	.1340643*	.06313
	8493-5 ºC	-.12163	.06313
	Inbred line-5 ºC	-.00949	.06313
Inbred line-5 ºC	8493-25 ºC	0.048886933	.06313
	You12-25 ºC	0.060847967	.06313
	Inbred line-25 ºC	.1334080*	.06313
	8493-15 ºC	0.116316267	.06313
	You12-15 ºC	0.0924176	.06313
	Inbred line-15 ºC	.1435506*	.06313
	8493-5 ºC	-.11215	.06313
	You12-5 ºC	.00949	.06313

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.

Chl a+b significance analysis

		Average difference	SD
8493-25 ℃	You12-25 ℃	.05976	.21640
	Inbred line-25 ℃	.30403	.21640
	8493-15 ℃	.21854	.21640
	You12-15 ℃	.14257	.21640
	Inbred line-15 ℃	.33735	.21640
	8493-5 ℃	-.5167500*	.21640
	You12-5 ℃	-0.144529833	.21640
	Inbred line-5 ℃	-0.1688995	.21640
You12-25 ℃	8493-25 ℃	-.05976	.21640
	Inbred line-25 ℃	0.244265533	.21640
	8493-15 ℃	0.158777567	.21640
	You12-15 ℃	.08281	.21640
	Inbred line-15 ℃	0.2775847	.21640
	8493-5 ℃	-.5765143*	.21640
	You12-5 ℃	-0.204294133	.21640
	Inbred line-5 ℃	-0.2286638	.21640
Inbred line-25 ℃	8493-25 ℃	-.30403	.21640
	You12-25 ℃	-0.244265533	.21640
	8493-15 ℃	-.08549	.21640
	You12-15 ℃	-0.161458033	.21640
	Inbred line-15 ℃	.03332	.21640
	8493-5 ℃	-.8207798*	.21640
	You12-5 ℃	-.44856	.21640
	Inbred line-5 ℃	-.4729293*	.21640
8493-15 ℃	8493-25 ℃	-.21854	.21640
	You12-25 ℃	-0.158777567	.21640
	Inbred line-25 ℃	.08549	.21640
	You12-15 ℃	-.07597	.21640
	Inbred line-15 ℃	.11881	.21640
	8493-5 ℃	-.7352919*	.21640
	You12-5 ℃	-0.3630717	.21640
	Inbred line-5 ℃	-0.387441367	.21640
You12-15 ℃	8493-25 ℃	-.14257	.21640
	You12-25 ℃	-.08281	.21640
	Inbred line-25 ℃	0.161458033	.21640
	8493-15 ℃	.07597	.21640
	Inbred line-15 ℃	.19478	.21640
	8493-5 ℃	-.6593218*	.21640

	You12-5 ºC	-0.287101633	.21640
	Inbred line-5 ºC	-0.3114713	.21640
Inbred line-15 ºC	8493-25 ºC	-.33735	.21640
	You12-25 ºC	-0.2775847	.21640
	Inbred line-25 ºC	-.03332	.21640
	8493-15 ºC	-.11881	.21640
	You12-15 ºC	-.19478	.21640
	8493-5 ºC	-.8540990*	.21640
	You12-5 ºC	-.4818788*	.21640
	Inbred line-5 ºC	-.5062485*	.21640
8493-5 ºC	8493-25 ºC	.5167500*	.21640
	You12-25 ºC	.5765143*	.21640
	Inbred line-25 ºC	.8207798*	.21640
	8493-15 ºC	.7352919*	.21640
	You12-15 ºC	.6593218*	.21640
	Inbred line-15 ºC	.8540990*	.21640
	You12-5 ºC	.37222	.21640
	Inbred line-5 ºC	.34785	.21640
You12-5 ºC	8493-25 ºC	0.144529833	.21640
	You12-25 ºC	0.204294133	.21640
	Inbred line-25 ºC	.44856	.21640
	8493-15 ºC	0.3630717	.21640
	You12-15 ºC	0.287101633	.21640
	Inbred line-15 ºC	.4818788*	.21640
	8493-5 ºC	-.37222	.21640
	Inbred line-5 ºC	-.02437	.21640
Inbred line-5 ºC	8493-25 ºC	0.1688995	.21640
	You12-25 ºC	0.2286638	.21640
	Inbred line-25 ºC	.4729293*	.21640
	8493-15 ºC	0.387441367	.21640
	You12-15 ºC	0.3114713	.21640
	Inbred line-15 ºC	.5062485*	.21640
	8493-5 ºC	-.34785	.21640
	You12-5 ºC	.02437	.21640

The mean followed by asterisk (*) in the same row differ significantly at P≤0.05, according to one-way ANOVA and LSD multiple comparisons.